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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR

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THE COLORIMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION AND ITS APPLICATIONS IN BACTERIOLOGY

PART III

WILLIAM MANSFIELD CLARK AND HERBERT A. LUBS

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SECTION XIV. ELECTROMETRIC AND COLORIMETRIC COMPARISONS

As indicated in a previous section, the solutions which must be dealt with in bacteriological investigations are diverse indeed and are not subject to a classification which facilitates systematic study of the indicator errors. We have considered it wise, therefore, to omit, for the present, detailed consideration of particular media, and have studied a considerable variety of solutions upon which we have made more than 400 electrometric measurements and many more colorimetric comparisons. These comparisons between the colorimetric and electrometric measurements are given in tables 4 to 19.

In these comparisons we shall consider the hydrogen electrode measurements as the standard, since they were made with an equipment and in a manner which has proved accurate and reliable. The details of the special electrometric methods used will be found in previous papers by Clark (1915 d) and Clark and Lubs (1916). Occasionally an electrometric determinator was made upon a solution not well adapted to hydrogen electrode measurements. Those familiar with the subject will recognize such a solution in Dorset's egg medium, for instance. In one or two other cases electrometric determinations with solutions in a P_H region where their buffer effect is small have been somewhat uncertain. In general, however, the electrometric measurements may be trusted to the second decimal of P_H if it be granted that the correct order of magnitude of liquid contact potentials has been properly determined (Clark and Lubs 1916). Where

the measurements are not to be so trusted the second decimal place will be omitted.

In the colorimetric comparisons we have purposely avoided, in this preliminary survey, some of the best colorimetric methods which we might have applied, such as the use of optical instruments of correct design, and we have used the somewhat wide interval of 0.2 P_H in the standard comparison solutions between which to interpolate. In these interpolations we have estimated only to the nearest 0.1. We have, furthermore, given our attention to colored and turbid solutions rather than to clear, colorless ones. In bringing various media within the range of the several indicators NaOH or HCl has been added, which generally produces a precipitate. This we have not filtered out but have studied the solutions with color and turbidity present. In short, our procedure while carried out with care and with exactly prepared standard comparison solutions has been such as we may reasonably expect the equipment of any laboratory to permit, and upon such material as that to which we must expect it to be applied. The electrometric measurements were made by one of us, the colorimetric measurements by the other and without any exchange of information which could influence the results. Except in two or three cases where obvious mistakes were involved and in two cases in which it was realized that no accurate measurements could be made, no data have been omitted because of disagreement between the values obtained with the two methods. The brackets found in the tables enclose values found for the solutions indicated at the left. Each value represents a determination on a separate sample.

In some instances a sufficient number of determinations were made with one kind of solution to justify a summary. Such summaries are given in tables 4, 5, and 6. In these tables we mean by "average deviation" the value obtained when the electrometric value was subtracted from the colorimetric value in each case and the arithmetical average of the + and − deviations was taken. Thus, in table 4 the average deviation for phenol red, comparator method, is + 0.03, and with dilution method, − 0.01, which means that if the electrometric determi-

nation were $P_H = 7.00$, the average colorimetric value in one case would be 7.03 and in the other 6.99. Since such averages might agree with the electrometric value, although the individual positive and negative deviations were large, we have given the "mean deviation," which is the mean of all deviations neglecting their sign. A ± following this shows that the P_H values obtained colorimetrically were greater in some cases than the electrometric value and in other cases less. When only + for instance, follows, it shows that the colorimetric P_H in every instance was greater than the electrometric P_H. The maximum and minimum deviations need no explanation except as to sign which follows the order given above.

In tables 8 to 9 we have included under "Remarks" only a rough indication of the depth of color in the solutions studied. As already mentioned, NaOH or HCl was added in many instances and this produced more or less turbidity. Only in the case of extreme turbidity, however, is this mentioned in "Remarks." In order to simplify the tables further, mention of the addition of acid or alkali will be omitted. *It will, therefore, be understood that to bring the* P_H *of certain solutions within the range of the indicators studied HCl or NaOH has been added although not mentioned in the tables.*

In the columns headed "Dilution" the values given were determined after dilution of 2 cc. of the solution to 10 cc. with distilled water. In all but a few cases such determinations were made without a comparator. In the columns headed "Comparator" the values given were determined without dilution and with the aid of a comparator according to Walpole's compensation method (see Journal of Bacteriology, ii, p.118 and 136). When determinations were made with a comparator after dilution of the solution the values given are placed *between* the other columns, unless dilution is mentioned under "Remarks".

There are contained in these tables numerous points which may be emphasized to better advantage in a discussion. A few of the more important will now be considered.

All the indicators studied are reliable for approximate determinations upon a wide variety of solutions, and, if good judgment

is used, they may be depended upon for approximate determinations even when the solution studied is highly colored and turbid. For precise determinations, however, each indicator must be used with caution in particular cases and some of them can not be depended upon.

TABLE 4

Deviations of colorimetric determinations from electrometric determinations of P_H in Dunham's solutions

NUMBER OF DETERMINATIONS	INDICATOR	COLORIMETRIC METHOD	DEVIATIONS OF P_H			
			Average	Mean	Maximum	Minimum
12	Brom phenol blue	Comparator	+0.10	0.10±	+0.16	+0.01
10		Dilution	+0.09	0.11±	+0.24	−0.02
17	Methyl red	Comparator	+0.21	0.21+	+0.35	+0.02
19		Dilution	+0.27	0.27+	+0.45	+0.13
3	Propyl red	Comparator	+0.09	0.10±	+0.15	−0.02
4		Dilution	+0.10	0.11±	+0.15	−0.02
10	Brom cresol purple	Comparator	±0.00	0.04±	−0.10	0.00
4	Brom thymol blue	Comparator	+0.08	0.12±	+0.14	+0.08
5		Dilution	+0.01	0.06±	−0.10	−0.02
14	Phenol red	Comparator	+0.03	0.06±	+0.14	−0.01
8		Dilution	−0.01	0.04±	+0.10	−0.01
8	Cresol red	Comparator	+0.07	0.07+	+0.13	+0.01
10	α-napthol phthalein	Comparator	−0.06	0.06±	−0.14	+0.02
5		Dilution	−0.05	0.05±	−0.11	+0.02
9	Thymol blue	Comparator	+0.07	0.07+	+0.11	+0.05
		Dilution	−0.05	0.06±	−0.15	−0.01

Let us consider each indicator in the order of the range of P_H which it covers.

Thymol blue in its *acid range* seems to be fairly reliable according to the limited number of determinations made with it (see table 8). It is especially gratifying to observe this because hitherto the zone of P_H covered has been without a brilliant and

at the same time reliable indicator. If our few determinations with thymol blue (acid range) are a good indication of its reliability it should be of great use in the study of yeasts,

TABLE 5

Deviations of colorimetric from electrometric values in P_H determinations of beef infusion, 1 per cent peptone broths

NUMBER OF DETERMINATIONS	INDICATOR	METHOD	DEVIATIONS			
			Average	Mean	Maximum	Minimum
7	Brom phenol blue	Comparator	−0.05	0.16±	−0.38	−0.01
6	Methyl red	Comparator	+0.10	0.11±	+0.28	0.00
4		Dilution	+0.08	0.08+	+0.18	0.00
4	Propyl red	Comparator	+0.08	0.08+	+0.18	0.00
2		Dilution	±0.00	0.00	0.00	0.00
10	Brom cresol purple	Comparator	−0.01	0.04±	±0.07	0.00
5		Dilution	−0.03	0.05±	−0.14	−0.01
14	Brom thymol blue	Comparator	−0.10	0.15±	−0.25	+0.03
5		Dilution	−0.10	0.12±	−0.26	+0.01
12	Phenol red	Comparator	−0.04	0.04±	±0.07	−0.01
8		Dilution	−0.06	0.06−	−0.12	−0.02
6	Cresol red	Comparator	−0.03	0.03−	−0.07	−0.01
3		Dilution	−0.06	0.06−	−0.11	−0.02
3	α-napthol phthalein	Comparator	−0.06	0.06−	−0.12	−0.02
5	Thymol blue	Comparator	−0.04	0.09±	+0.14	−0.01
3		Dilution	−0.01	0.03±	−0.06	−0.01
3	Phenol phthalein	Comparator	+0.03	0.07±	+0.14	−0.01
3	O-cresol phthalein	Comparator	+0.03	0.07±	+0.14	−0.01

molds, and acidophilic bacteria as well as for special purposes such as the study of the gastric contents.

Brom phenol blue has given considerable trouble because almost all the solutions tested with it have been turbid. This turbidity has introduced the error of dichromatic indicator solu-

TABLE 6

Deviations of colorimetric from electrometric determinations

NUMBER OF DETERMINATIONS	METHOD	DEVIATIONS IN P_H			
		Average	Mean	Maximum	Minimum
		Methyl red			
		Turbid cultures of *B. coli* in 1 per cent Witte peptone, 0.5 per cent K_2HPO_4+1 per cent various sugars			
36	Comparator	+0.03	0.04±	+0.13	±0.01
34	Dilution	+0.08	0.08±	+0.18	±0.01
		Turbid cultures of *B. coli* in 0.5 per cent Witte peptone, 0.5 per cent K_2HPO_4, 1 per cent to 3 per cent various sugars			
6	Comparator	+0.05	0.05+	+0.08	+0.01
11	Dilution	+0.10	0.10+	+0.28	+0.03

TABLE 7

Colorimetric and electrometric determination of the P_H of urines. Comparator used in all cases and screen with brom cresol purple in most cases

INDICATOR	P_H COLORIMETRIC	P_H ELECTROMETRIC
Methyl red	5.5	5.54
	5.3	5.38
	5.5	5.55
	5.3	5.33
Propyl red	5.7	5.77
	5.6	5.62
	6.0	6.01
Bromcre sol purple	6.4	6.39
	5.9	5.88
	6.5	6.67
	6.1	6.01
	6.0	6.04
	6.4	6.36
	5.7	5.62
	5.7	5.77
	5.5	5.54
Brom thymol blue	6.8	6.80
	6.5	6.67
	6.5	6.43
	6.4	6.38
Phenol red	6.8	6.80

tions which has been discussed in a previous section. Table 9, showing determinations with brom phenol blue does not, unfortunately, show any determinations made with the screened electric light. Some of these determinations are included in the summarized results of tables 4 and 5. A few measurements made with the mercury arc are shown in table 10.

TABLE 8

Comparison of P_H determinations made with thymol blue (acid range) and with hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	2.4 2.0	2.4	2.37 1.97
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color		1.9	1.77
0.2 per cent $NH_4H_2PO_4$, 0.02 per cent KCl, 0.02 per cent $MgSO_4$, 5 per cent sucrose, *Aspergillus* culture	Clear		1.8	1.76
0.1 per cent $(NH_4)_2HPO_4$, 0.02 per cent KCl, 0.01 per cent $MgSO_4$, 5 per cent sucrose, *Aspergillus* culture	Clear		1.9	1.86
Whey	Very turbid		2.7 1.9	2.56 1.64
"White vinegar"	Clear		2.3	2.36

Methyl red has given some puzzling results. Table 6 shows that it may be depended upon in the study of colon cultures on the media indicated and in the range of $P_H = 4.5 - 5.5$. On the other hand, methyl red has given more or less consistently high P_H values for various Dunham's solutions (table 4) and in many other cases (table 11). Superficially this appears to be more of a so-called "salt error" than a "protein error." Some of this inaccuracy with methyl red is doubtless due to the turbidity of media acidified to such a range of P_H.

TABLE 9

Comparison of P_H determinations made with brom phenol blue and with the hydrogen electrode. (Determinations made in daylight)

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
0.2 per cent $NH_4H_2PO_4$, 0.02 per cent KCl, 0.02 per cent $MgSO_4$, 5 per cent sucrose	Clear		4.3	4.3
1 per cent peptone, 1 per cent dried yeast	Moderate color, turbid	4.0 3.9 3.8		4.13 3.69 3.68
1 per cent peptone, 1 per cent dried yeast, 1 per cent K_2HPO_4	Moderate color, turbid	4.2		4.14
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color, turbid	4.1 3.9		3.95 3.41
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4	Moderate color, turbid	4.3 4.1 3.8		4.59 4.13 3.77
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color, turbid	3.9		3.71
1 per cent peptone, 0.5 per cent K_2HPO_4 (culture + HCl)	Very turbid		4.1 4.1	4.55
Cow feces extract (unfiltered)	Extremely colored and turbid		4.3	4.0
Silage juice	Green and very turbid		4.0 4.0	3.91 3.82
2 per cent egg white, pepsin digest	Turbid		3.9	3.59
Cider vinegar	Moderate color, not turbid		3.1	3.21
Apple juice	Moderate color, not turbid		3.8	3.76
Prune juice	Very dark, not turbid		4.2	4.12

Propyl red on the whole has given somewhat better results than methyl red in spite of the fact that it is chemically so similar and in spite of the fact that propyl red is comparatively so much less soluble that it precipitates on standing in the comparison solutions. We have used propyl red chiefly to cover a narrow range of P_H between those zones covered by methyl red and brom thymol blue, a zone about $P_H = 6.0$. Recently we have discovered that this same zone may be covered much more satisfactorily by the next indicator we shall discuss.

TABLE 10

Comparison of P_H determinations made with brom phenol blue and the hydrogen electrode. (Determinations in mercury are light)

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color, turbid	4.3	4.4	4.45
1 per cent peptone, 1 per cent dried yeast	Moderate color, turbid	4.0	4.0	3.98
		3.8		3.72
		3.7		3.65
		3.9		3.80
		3.9		3.84
Liver infusion, 1 per cent peptone	Extremely dark, and turbid	3.7	3.8	3.70
1 per cent peptone, 1 per cent dried yeast, 1 per cent glucose, culture	Very turbid	4.0	4.0	3.98

Brom cresol purple exhibits the dichromatism discussed in a previous section and may produce the same confusion which has rendered brom phenol blue so hard to deal with. This indicator on the other hand lends itself beautifully to determinations made in the screened electric light. So used, its indications in Dunham's solutions (table 4), beef infusion media (table 5), urine (table 7) and in several other cases (table 13) are very satisfactory. This is important, for the range of P_H which may be studied with this indicator is one of great bacteriological importance. The brilliant red color of brom cresol purple in the screened

TABLE 11

Comparison of P_H determinations made with methyl red and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Turbid	4.8	4.7	4.49
1 per cent yeast, 1 per cent peptone	Turbid	4.8	4.8	4.55
			5.3	5.32
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugar (cultures)	Very turbid	5.1	4.9	4.94
			5.2	5.18
			5.2	5.15
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4	Very turbid	5.5	5.4	5.25
Veal infusion, 1 per cent peptone	Very turbid	4.9		4.59
Liver infusion, 1 per cent peptone	Very dark, 5–10 dilution	5.4	5.2	5.31
			5.3	5.31
		4.8		4.67
			4.8	
Beer wort	Very dark	5.1	4.9	4.91
Carrot juice	Moderate color	5.5	5.4	5.21
Cucumber juice			5.1	5.08
Apple juice	Dark color		5.1	5.02
String bean juice	Moderate color	5.5	5.3	5.23
Prune juice	Dark color	4.8	4.5	4.41
		5.1	4.7	4.80
		5.3	5.1	5.08
Banana juice	Extremely turbid, diluted 2–10		4.7	4.62
Whey	Very turbid, diluted 2–10		5.0	4.69
Cow feces extract (unfiltered)	Extremely turbid		4.3	4.1
2 per cent egg white partial pepsin digest	Very turbid		5.0	4.9
2 per cent egg white full pepsin digest	Turbid		4.7	4.49

light has an advantage over the blues and greens of the indicator next in order, and should, when possible, be used in preference to it. The screened light is chiefly useful in the more acid region.

TABLE 12

Comparison of P_H determinations made with propyl red and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC
		Dilution P_H	Comparator P_H	P_H
1 per cent peptone, 0.5 per cent K_2HPO_4, 3 per cent various sugars (cultures)	Very turbid	6.2	6.0	6.07
		5.6	5.5	5.51
		5.9	5.8	5.81
			5.8	5.59
		6.0	5.8	5.84
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Moderate color	6.1	6.0	5.91
1 per cent yeast, 1 per cent peptone	Moderate color	5.3		5.32
		5.7		5.60
1 per cent yeast, 1 per cent peptone, 1 per cent K_2HPO_4	Moderate color	6.3		6.12
1 per cent yeast, 0.3 per cent Liebig's extract	Moderate color	5.9		5.80
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4	Moderate color	6.2		5.97
		5.5		5.25
Beer wort	Very dark	6.1	6.0	5.92
10 per cent gelatine, 0.5 per cent Liebig's extract	Moderate color		6.0	6.04
Hay infusion	Moderate color	5.9	5.7	5.81
Irish potato juice	Black	6.1	6.2	6.06
Apple juice	Dark		5.7	5.65
Sweet potato juice	Dark	5.8	5.7	5.80

Brom thymol blue. In the conduct of this indicator, whose solutions exhibit no appreciable dichromatism, may be discerned some evidence that a blue indicator is not particularly well adapted to colorimetric determinations with colored solutions. The data in table 14 indicate that while very good determinations have been made with this indicator, the results are somewhat uncertain. This has been especially noted in the

study of beef infusion media (table 5). There is no difficulty in obtaining good approximate results with this indicator, but its color changes, especially in the greens, are deceptive and must be judged with caution if accurate data are desired. As already suggested the absorption bands of colored media themselves may

TABLE 13

Comparison of P_H determinations with brom cresol purple and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 1 per cent yeast	Moderate color	6.7		6.73
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	6.5	6.5	6.43
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugar	Turbid cultures		5.6	5.63
		6.4	6.5	6.58
		6.4	6.5	6.61
Cow feces extract	Unfiltered, extremely turbid		6.8	6.9
			6.8	6.7
			6.8	6.7
Hay infusion	Moderately dark		5.9	5.81
			5.9	5.99
Liver infusion, 1 per cent peptone	Very dark, 5–10 dilution, 2–10 dilution		6.2	6.19
			6.2	
Veal infusion, 1 per cent peptone	Moderate color		6.3	6.21
			5.3	5.26
10 per cent gelatine, 0.5 per cent Liebig's extract	Moderate color	6.0	6.0	6.04

cause confusion in the judgment of a blue indicator even when the compensation method is used. At present we believe that brom cresol purple and phenol red may be used in sufficiently extended ranges so as to make unnecessary any extensive use of brom thymol blue.

Phenol red, which we propose as an abbreviated name for phenol sulfon phthalein, we have found to be very reliable. In

TABLE 14

Comparison of P_H determinations made with brom thymol blue and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glycerine	Turbid cultures	6.7	6.6	6.74
		6.7	6.5	6.69
		6.3	6.2	6.28
		6.1	6.0	6.09
		6.5	6.3	6.53
		6.4	6.3	6.27
		6.3	6.2	6.32
1 per cent yeast	Moderate color	6.3		6.80
1 per cent yeast, 1 per cent peptone	Moderate color	6.6		6.77
			6.5	6.73
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl, 0.1 per cent K_2HPO_4	Moderate color	6.7	6.7	6.63
			6.2	6.43
			6.9	7.05
1 per cent peptone, 1 per cent yeast, 1 per cent K_2HPO_4	Moderate color	7.2		7.15
		6.9		6.91
		6.7		6.68
		6.5	6.4	6.42
		6.2		6.12
Beef infusion (double strength), 2 per cent peptone	Dark color		6.8	6.14
		6.0	6.0	6.00
			6.0	6.39
			6.3	6.62
		7.0	6.9	7.07
Liver infusion, 1 per cent peptone	Very dark, 5-10 dilution		6.7	6.89
Beer wort	Very dark	6.9	6.7	6.84
Hay infusion	Moderate color	6.9	6.9	6.97
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color		6.0	6.04
Vermont maple sirup	Very dark and thick	6.8	6.9	6.75
Extract cow feces (unfiltered)	Extremely turbid and colored		7.1	7.1
			6.8	6.9
			6.6	6.7
			6.8	6.6
			6.6	6.7

TABLE 14—*Continued*

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
Whey	Very turbid, 2-10 dilution		6.4 6.4	6.55 6.44
2 per cent egg white (partial pepsin digest)	Turbid		6.8	7.1
Carrot juice	Moderate color	6.6	6.5	6.43
String bean juice	Moderate color	6.9	7.0	7.15
Prune juice	Dark color	6.6	6.6	6.46
Sweet potato juice	Moderate color	6.7	6.6	6.80
Beet juice	Very dark	6.2	6.2	6.07

very few instances has it given results which could be called inaccurate. The data found in tables 4, 5, 7, and 15 need no detailed discussion. This indicator is invaluable because it is useful in the region of true neutrality. Although its praise has been sung by others, we believe that the data we here present are the first which show in any extensive manner the agreement between colorimetric determinations made with this indicator and electrometric determinations.

Cresol red. What has been said of phenol red appears to hold true for cresol red, although we have not studied it so extensively. It is very important to note that cresol red extends the range which may be accurately studied colorimetrically appreciably further into the alkaline region than phenol red, and that its color changes, from clear yellow to brilliant red, are preferable to those of the previously used α-naptholphthalein whose intermediate colors are rather muddy. Data obtained with cresol red will be found in tables 4, 5, and 16.

α-naptholphthalein, which we studied before we realized the usefulness of cresol red in the same region of P_H, has proved reliable but not so satisfactory as cresol red. Several determinations with α-naptholphthalein will be found in tables 4, 5, and 7.

Thymol blue (alkaline range) is one of those indicators with which we again encounter difficulties that appear to attend the use of a yellow-blue color change. On the whole, however, thymol blue has proved more satisfactory than its brom deriva-

TABLE 15

Comparison of P_H determinations made with phenol red and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glucose	Moderate color	7.1	7.0	6.95
1 per cent peptone, 1 per cent K_2HPO_4, 1 per cent yeast	Moderate color	7.5		7.58
		7.2		7.15
		6.9		6.91
		7.7		7.68
1 per cent peptone, 1 per cent yeast	Moderate color	7.4		7.45
		8.1		8.24
			6.7	6.73
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color	7.1		7.18
		7.4		7.57
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glycerine	Very turbid cultures	7.2		7.20
		7.6		7.62
		7.2	7.2	7.18
		7.3	7.3	7.22
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugars	Very turbid cultures		7.1	7.10
			7.7	7.77
			7.7	7.78
			7.7	7.70
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugars	Moderate color	7.1	7.1	7.10
			7.6	7.65
			7.7	7.67
			7.4	7.45
			7.0	7.07
			7.5	7.51
1 per cent peptone, 0.5 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	7.4		7.39
		8.3		8.30
		6.9		7.05
McConkey's bile salt medium	Moderate color	7.3	7.3	7.45
			8.4	8.43
Beef infusion, 1 per cent peptone, 0.5 per cent NaCl, 1 per cent glucose (overheated)	Deep color		7.0	6.83
			7.0	6.72

TABLE 15—Continued

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
0.3 per cent Liebig's extract, 1 per cent peptone, 0.5 per cent NaCl, 0.1 per cent K_2HPO_4	Moderate color	7.1 7.0	7.1 7.0	7.06 6.99
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4	Moderate color	7.1		7.17
Liver infusion, 1 per cent peptone	Very dark, dilution 5–10		6.9 7.3 8.1	6.89 7.32 8.13
Veal infusion, 1 per cent peptone	Moderate color	7.0 7.7	7.0 7.5	6.98 7.66
10 per cent gelatine, 0.5 per cent Liebig's extract	Moderate color	7.3 7.5	7.3 7.4	7.41 7.58
Dorset's egg medium before coagulation	See text	7.2-7.6		7.4
Hay infusion	Moderate color	6.9	6.9 8.1	6.97 8.13
Beer wort	Very dark		7.8	7.79
Cow urine	Moderate color		7.4	7.4
Cow feces extract (unfiltered)	Extremely turbid		7.3	7.1
Sweet potato juice	Moderate color	7.4	7.4	7.38
Carrot juice	Moderate color	7.8	8.1	7.85
White potato juice	Black!	7.7	7.9	7.71
String bean juice	Moderate color	7.7	8.1	8.08
Prune juice	Moderate color	7.7	7.8	7.80
Beet juice	Black!	7.7		7.92
0.1 per cent $(NH_4)H_2PO_4$, 0.02 per cent KCl, 0.01 per cent $MgSO_4$, 5 per cent sucrose; after sterilization	Clear		7.05	7.08

tive, brom thymol blue, and we place as much confidence in it as we do in phenolphthalein which covers most of the same P_H zone. Determinations with thymol blue are given in tables 4, 5, and 18. We may call particular attention to a point frequently observed. When making determinations with thymol blue and

simultaneously with phenolphthalein or o-cresol phthalein, which are one-color indicators, we have found, when the solution under examination is colored, that the two-color indicator, thymol blue, is preferable. The approximate P_H, at least, may be discerned with much greater ease than when the one-color indicators are used. This has appealed to us forcibly.

TABLE 16

Comparison of P_H determinations made with cresol red and with the hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	7.4	7.4	7.39
		8.3	8.3	8.30
1 per cent peptone, 0.5 per cent K_2HPO_4 (cultures)	Very turbid		7.7	7.77
		7.7	7.7	7.78
		7.7	7.6	7.70
		7.7	7.7	7.70
Veal infusion, 1 per cent peptone	Moderate color, very turbid	8.4	8.6	8.49
			7.0	6.98
			7.7	7.66
Liver infusion, 1 per cent peptone	Very dark, turbid	7.3		7.32
		8.1	8.1	8.13
Hay infusion	Moderate color	8.0	8.1	8.13
		8.2	8.3	8.40
McConkey's bile salt medium	Moderate color		7.3	7.45
		8.3	8.4	8.43
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color	7.4	7.4	7.58
2 per cent egg white, partial tryptic digest			8.5	8.4

Ortho cresol phthalein is useful as a supplement to thymol blue. Determinations made with it will be found in tables 5 and 19.

Phenolphthalein in some dozen tests made with it gave values close to those indicated by ortho cresol phthalein but with the advantage in favor of the cresol compound. We have aban-

doned phenolphthalein almost entirely for P_H determinations and whenever there has been occasion to use it, whether in determinations of P_H or in titrations, we have preferred its homologue, the more brilliant ortho cresol phthalein.

A few particular observations deserve notice. Determinations with whey taken from a cheese vat, with banana juice, cow feces, silage juice, and Dorset's egg medium, are examples of

TABLE 17

Comparison of P_H determinations made with α-napthol phthalein and with the hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC
		Dilution P_H	Comparator P_H	P_H
1 per cent dried yeast, 1 per cent peptone	Moderate color	8.1		8.24
1 per cent dried yeast, 1 per cent peptone, 1 per cent K_2HPO_4	Moderate color	8.4		8.44
		7.5		7.48
		7.7		7.68
		7.5		7.58
		8.0		7.99
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Moderate color	8.1	8.2	8.08
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color	8.1		8.47
Beer wort	Very dark	7.6–7.7		7.79
String bean juice	Dark		8.0	8.08
Prune juice	Dark		8.0 8.1	8.14
			8.1 8.1	8.24
Irish potato juice	Black		8.4 8.4	8.57

solutions which have been studied which were so turbid that any colorimetric determination appeared hopeless. By the use of dilution, or a comparator, or both, and by the use of the brilliant indicators we have described, reasonably good determinations were made with these solutions. Dorset's egg medium we could only place between 7.2 and 7.6 (see table 15). Accurate electrometric determinations with this medium are extremely difficult. Two determinations with freshly platinized electrodes gave 7.38 and 7.41, and the average of five deter-

minations with old and new electrodes was 7.36. If the rounded value 7.4 is taken for the electrometric determination the colorimetric limits 7.2 to 7.6 which we found are in agreement to a surprising degree, when the dense turbidity and color of this medium is considered.

TABLE 18

Comparison of P_H determinations made with thymol blue (alkaline range) and the hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 1 per cent yeast, 1 per cent K_2HPO_4	Moderate color	8.9 8.4		8.91 8.44
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Moderate color	9.4	9.5	9.60
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glucose (culture)	Extremely dark and turbid	8.5		8.54
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color	8.4	8.4	8.60
Hay infusion	Dark		9.2	9.40
Veal infusion, 1 per cent peptone	Moderate color		9.2	9.24
2 per cent egg albumin, partial tryptic digest			8.5	8.4
Irish potato juice	Black		8.9 9.0 9.4 9.4	8.97 9.44
Carrot juice	Moderate color		8.6 8.9 8.9 9.2	8.81 9.27
Beet juice	Very dark		8.4 8.7 8.8	8.57 8.75
Sweet potato juice	Moderate color		8.7 8.8	8.75
Prune juice	Very dark		9.5	9.44

Much less difficult were determinations made with such solutions as oxidized potato extract, liver infusion, deep red overheated sugar bouillons and green silage juice, the coloration of which made these solutions look almost black when in bulk. In fact, we have experienced much less difficulty with color than with turbidity.

We feel certain that had we given more attention to clear and colorless solutions the colorimetric and electrometric comparisons would have shown better agreement in many cases. In other cases we might have been able to show with greater clarity consistent discrepancies such as have appeared in tests of Dunham's solutions with methyl red. We believe, however, that a systematic investigation of "protein" and "salt" errors had

TABLE 19

Comparison of P_H determinations made with O-cresol phthalein and with hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
Dunham's solution	Moderate color		8.6 9.5	8.65 9.40
1 per cent dried yeast	Moderate color		8.8	8.93
1 per cent dried yeast, 1 per cent peptone	Moderate color		8.8 9.1	8.92 9.27
1 per cent dried yeast, 1 per cent peptone, 1 per cent K_2HPO_4	Moderate color		8.3 8.9	8.44 8.91
Beer wort	Very dark	8.4		8.55
Prune juice	Dark		9.3	9.44
Sweet potato juice	Moderate color		8.7	8.75
Beet juice	Black	8.4		8.57
String bean juice	Moderate color		8.5	8.63
Hay infusion	Moderate color		9.2	9.4
10 per cent gelatine, 1 per cent peptone			8.4	8.60

best be left to more intensive investigation of particular solutions. Our present purpose has been to subject the indicators and the simple crude methods which we have described to as severe trials as could reasonably be imposed, and it may again be emphasized that we have *not selected* solutions which are particularly well adapted to colorimetric determinations, we have not adjusted the salt content of the solutions, we have not filtered off any precipitates which were found or produced by the

addition of acid or alkali, and we have sought only to attain such accuracy as could be attained in less time than that required for the electrometric determinations. The results in certain instances may be regarded as unsatisfactory by those who seek to make the colorimetric method precise, but in our opinion they are, with few exceptions, quite close enough for all bacteriological work which does not require the precision of electrometric determinations.

SECTION XV. ADJUSTMENT OF CULTURE MEDIA

The adjustment of the P_H of culture media is one of the most obvious applications of the colorimetric method as was suggested by one of us in a former paper (Clark (1915 e)). It has taken us some time to complete the investigations which were promised in this former paper, and, in the meantime, Hurwitz, Meyer and Ostenberg (1915, 1916) have to some extent anticipated our treatment of this particular subject.[19] Attention, however, may be called to the fact that these authors did not make comparisons between colorimetric and electrometric measurements upon which judgment of the accuracy of colorimetric measurements should at present be based.

The procedures suggested by Hurwitz, Meyer and Ostenberg, it seems to us, have been unnecessarily complicated. In the first place they appear to have avoided by all possible means dilution of the solutions in testing for P_H, not realizing how inappreciable is the change in P_H on moderate dilution of the solutions studied. They have suggested the use of Standardized

[19] Anthony and Ekroth (1916), discussing the adjustment of culture media in a recent paper, have made several statements concerning the theory involved with which we must disagree. We are not aware that the hydrogen electrode has ever been used in *conductivity* measurements of hydrogen ion concentration. Points of inflexion in titration curves certainly do not indicate the true neutral point, nor does the nearness of an indicator's "point" of change to this point of inflexion determine its suitability for a particular medium. We regret that the interesting data presented in this paper by Anthony and Ekroth are discussed in so many details from points of view with which ours are at variance, and that some of the work of this and other laboratories has been erroneously interpreted.

N/20 and N/1 alkali, which is of course necessary in adjustments to Fuller's scale; they have proposed a solution which cannot be accepted without more investigation than they record, namely the indicator mixed with the alkali solution used in titrating and they have burdened the subject with what we believe to be too elaborate and to some extent inaccurate stoichometrical relations. It may also be noted that these authors restricted their attention to adjustments which could be made with phenol sulfon phthalein (phenol red). It is undoubtedly true that the requirements of many bacteria, especially those which live in the blood stream, are met when media are adjusted to the blood reaction or to neutrality. But such a consideration has little weight with the plant pathologist or with one who must study the organisms of cheese, silage, wines, beers, soils, sewage, the acid loving yeasts and molds, the alkali enduring organisms or the metabolism of organisms under different environments. We consider it unwise therefore to confine adjustments to any particular range of P_H and we shall refrain from suggesting any "standard" P_H whatsoever, believing that this is a subject which should be left to separate researches and that the adoption of a standard in any particular line of investigation should be based upon experimental data rather than upon general considerations. We may however again emphasize the wide range of usefulness of the set of indicators we are describing.

The procedure we recommend may be illustrated as follows.

Having the colorimetric equipment described in section XII there is needed in addition a *roughly* standardized N/I NaOH solution relatively free from carbonate and a more dilute solution made by an *exact* 1–10 dilution of the first. This may be called the N/10 NaOH solution.

A standard beef infusion medium was to be given an initial reaction of P_H 7.4. After the original infusion had been properly diluted, heated and filtered, one per cent peptone was added. The mixture was kept warm for fifteen to twenty minutes with occasional stirring and then filtered. When cooled under a tap to room temperature the solution was made up to a known volume, less than the final volumes, and the reaction was adjusted

roughly after titrating 5 cc. with the N/10 NaOH solution in the presence of phenol red until it was judged that P_H 7.4 was reached. There were required for this 0.85 cc. N/10 NaOH. Hence to one litre of the solution were added 17 cc. of the N/1 NaOH solution. The medium was now ready for the *preliminary heating, at or near the final reaction.* Heating for fifteen minutes in the autoclave at 15 pounds pressure brought down a precipitate which was filtered out. The reaction as then determined colorimetrically (comparator) was somewhat more than 7.3. For most purposes this would have been sufficiently close to the value desired, but an attempt was made to adjust the reaction more precisely.

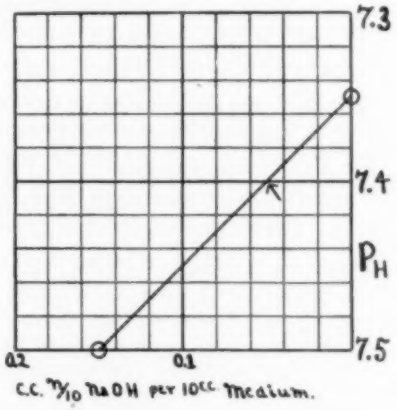

FIG. 8. PORTION OF TITRATION CURVE USED IN ADJUSTMENT OF A BEEF INFUSION MEDIUM

For a test 10 cc. were taken and readings were made with the aid of a comparator. On the addition of 0.15 cc. N/10 NaOH P_H = 7.5 was reached. This and the initial value, which was judged to be 7.35, were plotted as in figure 8. Interpolating, it was found that 7.4 would have been reached on the addition of 0.05 cc. N/10 NaOH to the 10 cc. of medium. Hence there were added to a litre of the solution 0.5 cc. N/1 NaOH. A colorimetric check showed that the desired reaction of 7.4 P_H had been

given to the medium. An electrometric determination however showed that the true reaction was $P_H = 7.47$.[20] The solution was now contaminated so it was resterilized after its volume had been brought to the desired final point. After this the reaction found colorimetrically was 7.3 and electrometrically 7.36.

This procedure may serve to illustrate *in outline* what we may suggest as a provisional method. It will perhaps need modification with special media, and in special cases. Hand in hand with the adjustment of the reaction of media must go a consideration of the substances precipitated by change in P_H, a consideration of the manipulation which is permissible, a consideration of the amount of reheating which is permissible, and a consideration of the change in P_H during sterilization. These constitute a few items of a very large subject which, we cannot treat in this paper in any adequate manner. Some of the diffi-

[20] These are about the maximum errors which we have observed with this indicator in beef infusions. We quote this particular example, however, to indicate that great accuracy cannot always be assured. Although the point attained before sterilization was 7.4, exactly that desired, when the colorimetric method alone was used, as in the studies of Hurwitz Meyer and Ostenberg, the error of 0.07 P_H was discovered when the electrometric measurement was considered as the true value.

It may be illuminating to those who are more familiar with "percentage titratable acidities" to note that in this particular solution this error amounts to about 0.07 per cent in "titratable acidity." In less strongly buffered solutions a like error in P_H would correspond to a much smaller error in "titratable acidity." In 0.3 per cent Liebig's extract, 1 per cent Witte peptone it would amount to about 0.03 per cent in "titratable acidity."

It cannot be emphasized too strongly that the reference point in determining "titratable acidities" of culture media is in reality but a P_H point indicated by the tint of phenol phthalein, that the particular tint (or particular P_H tacitly assumed) adopted by different workers varies, that the particular tint which is sometimes assumed is that at which phenol phthalein is least sensitive, and that without a proper standard comparison solution of known P_H value the same worker can hardly avoid errors of 0.1 P_H in establishing his *reference point* for "titratable acidity." Consequently "titratable acidities" which are apparently identical may vary much more widely when translated into an accurate titration curve than will the corresponding P_H values when determined by even crude methods. *Different* media adjusted to the same degree of "titratable acidity" may vary enormously in P_H. Since the publication of the paper "The 'Reaction' of Bacteriologic Culture Media" numerous instances have come to our notice of the errors, the inadequacy, and the confusion of the titrimetric method of adjusting media.

culties involved may be partly obviated by adjustments *after* sterilization such as Deelman used (see also Hurwitz, Meyer and Ostenberg (1916)). It may be suggested, however, that in a great many cases adjustment by the most direct and simple procedure will insure a medium more constant in composition and more constant in true reaction than that obtained by complex manipulation or adjustment on the old scale. We do urge that the most simple and direct procedures be given a trial before more elaborate ones creep into "standard methods." A reason for this will be discussed in section XVIII.

For the adjustment of the common solid media the following considerations may be offered. Gelatine media may be kept liquid at moderate temperatures and adjusted like liquid media. Pure agar has practically no buffer effect in the P_H ranges usually employed. Its addition to a medium should not appreciably affect the P_H of a solution, and consequently agar media may be adjusted before the addition of the agar. (High temperatures, over 40°C. should be avoided in using indicators since we have no data for these temperatures.) What effect will ensue when gel formation takes place in gelatine or agar media we may not venture to guess. If marked changes in *apparent* P_H as shown by indicators are observed, it may be found better by empirical tests to adjust to the *apparent* P_H desired as observed in the solidified medium. What interpretation other than a purely empirical one may then be put upon the indication we do not know. We are certain however that it will be quite as reliable if taken at face value as any "titratable degree of reaction" the determination of which involves implicitly the determination of a reference P_H point by means of an indicator.

Reduced to its lowest terms the colorimetric method of adjusting the "reaction" of culture media is a return to the older simple method of adjusting to a given tint of an indicator as with litmus, but with more reliable indicators, a wider choice of indicators, a logical scale of "reaction" and a clearer conception of the ends desired in adjustment. The method contains no sources of error not inherent in the titrimetric method and it avoids many sources of error which are inherent in the titrimetric method.

SECTION XVI. CONDUCT OF INDICATORS IN ACTIVE CULTURE MEDIA

It is well known that many compounds are reduced in the presence of active growths of bacteria. Reduction of colored compounds is generally accompanied by a color change. This has no *direct* connection with the color change of indicators due to alteration of P_H. Furthermore some indicators are not only reduced but fundamentally altered or destroyed by bacterial action. Therefore the conduct of an indicator when left exposed to bacterial action may be complex.

It is not to be denied that such complex conduct may furnish valuable information, just as the complex picture of numerous growth characteristics is significant to a trained eye. Nevertheless the use of indicators as indicators of P_H should be kept distinct from their other uses; and, when used as indicators of P_H, they should be introduced only at the time of the test unless used to indicate roughly the reaction. We emphasize this distinction because some have imagined that in the colorimetric measurements we are discussing, the indicators are made a part of the medium.

We have confirmed with cultures of *B. coli* Fred's (1912) observation with denitrifying organisms, that methyl red may be destroyed by bacterial action. Indeed this indicator should not be allowed to stand for more than a few minutes in active cultures. The sulfon phthalein indicators are of a very much more resistant nature. Some preliminary tests indicate that these indicators may be used to advantage in replacing other indicators which are now used in making indicator media.

We may emphasize particularly the promising value of brom cresol purple to replace the inconstant and often impure litmus or azolitmin widely used for "litmus milk." It imparts to milk a bluish grey-green color which changes on sterilization of the milk only to the extent that might be expected from the consequent change in P_H. As acid fermentations take place the color becomes yellow in easily distinguished gradations, and when alkali formation occurs a distinct blue color is imparted to the milk.

SECTION XVII. THE REACTION OF IMPORTANT SOLUTIONS

When attention is transferred from the analytically determined acid or alkali content or the so called titratable acid or alkali of solutions to the hydrogen ion concentration, some confusion is inevitable unless one has some idea of the position on the P_H scale of familiar solutions. In figure 9 are shown some of these relations. The figure is supplemented by tables 20 to 24 in which are given compilations of the P_H values of body fluids, fruit and plant juices, miscellaneous solutions and some optimal agglutination points and limiting reactions for bacteria. These compilations are given merely to help orient the true reactions of important and familiar solutions and for their suggestive value. No attempt has been made to cover the voluminous literature in detail or to present "weighted values."

SECTION XVIII. GENERAL DISCUSSION

It would be an injustice to the subject, if, after discussing its importance, its limitations and its advantages in bacteriology, we failed to indicate how the true field of usefulness of the colorimetric method is revealed by a consideration of certain broad aspects of the influence of hydrogen ion concentration.

Without fear of contradiction we may state that the hydrogen ion concentration influences the condition in solution of every substance with acidic or basic properties—native proteins and their products of hydrolysis, amines and amids, carboxyl, sulfonic and phenolic compounds, even alcoholic compounds, as well as very many of the inorganic compounds. Largely as a consequence of this the hydrogen ion concentration of a solution has a part in determining effective solubilities and the dispersion of colloids, in determining tautomeric equilibria, and in one way or another in governing the activity of catalists such as the hydrolytic enzymes and the oxidases. One or another of these effects, induced directly or perhaps very indirectly by the hydrogen ion concentration of a medium, must impress bacterial life.

Such a generalization, however, would be entirely misleading if not tempered by a proper appreciation of proportion. Rarely

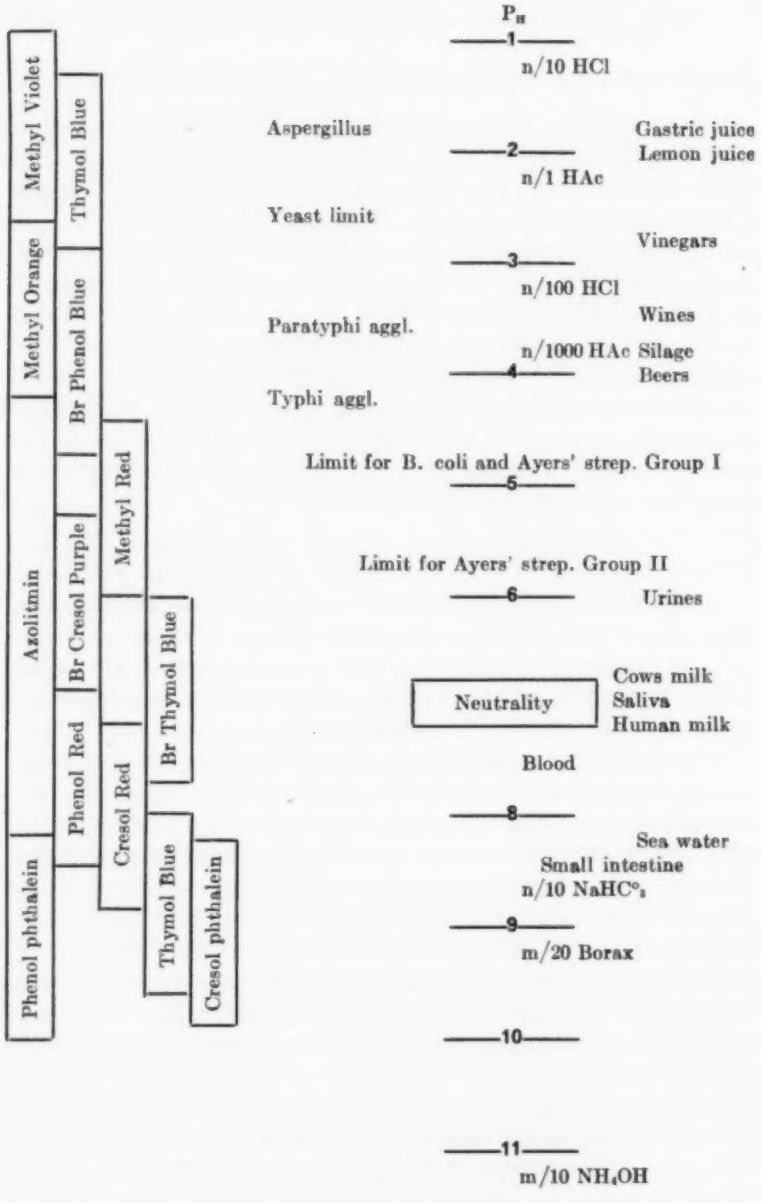

FIG. 9. THE P SCALE SHOWING THE POSITION OF IMPORTANT SOLUTIONS AND INDICATOR RANGES.

TABLE 20

True reactions of body fluids

FLUID	P_H	AUTHORITY
Blood	7.4	(See Michaelis (1914 b)
Urine	6.0[21]	(See Henderson and Palmer (1912) and Blatherwick (1914))
Saliva	6.9[22]	Michaelis and Pechstein (1914)
Gastric juice (adult)	0.9–1.6	Menton (1915)
Gastric juice (infant)	5.0	Hess (1915)
Pancreatic juice (dog)	8.3	Auerbach and Pick (1912)
Small intestinal contents	8.3	Auerback and Pick (1912)
Small intestinal contents (infant)	3.1	McClendon (1915)
Bile from liver	7.8	Okada (1915)
Bile from gall bladder	5.3–7.4	Okada (1915)
Perspiration	7.1	Foà (1906)
Perspiration	4.5[23]	Clark and Lubs
Tears	7.2	Foà (1906)
Muscle juice (fresh)	6.8	Michaelis and Kramsztyk (1914)
Muscle juice (autolyzed)	Variable	Michaelis and Kramsztyk (1914) (see Morse (1916))
Pancreas extract	5.6	Long and Fenger (1916)
Peritoneal fluid	7.4	Foà (1906)
Pericardial fluid	7.4	Foà (1906)
Aqueous humor	7.1	Foà (1906)
Vitreous humor	7.0	Foà (1906)
Cerebrospinal fluid	7.2	Foà (1906)
Cerebrospinal fluid	8.3	Hurwurtz & Tranter (1916)
Amniotic fluid	7.1	Foà (1906)
Amniotic fluid	8.1	Löb and Higuchi (1910)
Milk (human)	7.0–7.2	(See Clark (1915 b))
Milk (cow)	6.6–6.8	(See Clark (1915 b))
Milk (goat)	6.6	Foà (1906)
Milk (ass)	7.6	Foà (1906)

[21] This is a purely "general average." The reaction of urine varies considerably.

[22] The true reaction of saliva is worthy of more extensive investigation from the bacteriological point of view. The value $P_H = 6.9$, which is given by Michaelis and Pechstein, is almost exactly that of absolute neutrality. If applied at 37°C. it is more alkaline than acid, since the neutral point at 37° is about $P_H = 6.8$. Several salivas which we have tested colorimetrically were found to be 6.8–6.9, yet these were distinctly acid to both red and blue litmus paper, and would therefore be called "acid" salivas. It is not to be denied that, so far as the action upon the constituents of the teeth is concerned, the relation of this reaction to absolute neutrality is of little consequence. Rather must it be

TABLE 21

True reactions of miscellaneous solutions

SOLUTION	H	AUTHORITY
Soil extracts	4.5–8.7[24]	Gillespie (1916)
Sea water	8.0–8.4	Palitzsch (1911 a)
Mineral waters	6.5–7.0	Michaelis (1914 b)
Flour extract	6.0–6.5	Jessen-Hansen (1911)
Tan liquors	3.5–4.7	Wood Sand and Low (1911)
Vermont maple sirup	6.8	Clark and Lubs
Beers	3.9–4.7	Emslander (1914)
Wines	2.8–3.8	Paul (1914)
Cider vinegar	3.1	Clark and Lubs
"White vinegar"	2.6	Clark and Lubs
Corn silage juice	3.7[25]	Clark and Lubs
Human feces (adults')	7.1–8.8	Howe and Hawk (1912)
Human feces (infants')	6.0–7.0	(See Michaelis (1914 b)·)
Cow feces	6.6–7.2	Clark and Lubs
Snail lymph	9.0	Foà (1906)

related to the solubilities of the constituents of the teeth at different P_H. Nevertheless this neutral reaction of so-called "acid" salivas is a good illustration of the confusing connotations which have been attached to the term acidity.

[23] The value of $P_H = 7.1$ was obtained by Foà (1906) on perspiration whose flow was stimulated by a hot air bath and from skin which had been washed with alkali to remove the fatty acids. These fatty acids, it has been claimed, are not normal constituents of the perspiration but are excreted by the sebaceous glands. Whatever may be the value of Foà's determination for the student of the mechanisms by which the body reactions are regulated it is of little interest to the bacteriologist when compared with the importance of the reaction of the perspiration as it normally occurs. It has been very commonly held that the reaction of the perspiration is variable but generally acid. Park and Williams (1910) state "The secretions of the sebaceous glands appear to be little, if at all, bactericidal, but the perspiration, on account of its acidity, is slightly so."

Three samples which we collected from clean normal skin had a reaction of about $P_H = 4.5$. Whether this is extreme and exceptional or not remains for others to determine. We may however note that such a reaction will doubtless tend to keep dormant many bacteria even if it does not exert a distinct bactericidal action.

[24] This wide variation in the acidities of soil extracts cannot fail to indicate some wide differences in the effective acidities of the soils themselves. These observations by Dr. Gillespie, which are the most enlightening data on the mooted question of soil acidity, are of profound importance to soil bacteriology.

[25] This acidity of silage juice is considerably higher than that attained in cultures of any of the streptococci we have yet studied and is practically the P_H that some of our unpublished researches have shown the bacteria of the Bul-

TABLE 22

True reaction of fruit and plant juices

SOLUTION	P_H	AUTHORITY
Lime juice	1.7	Patten and Mains[25]
Lemon juice	2.2	Patten and Mains[25]
Cherry juice	2.5	Patten and Mains[25]
Grapefruit juice	3.0–3.3	Patten and Mains[25]
Orange juice	3.1–4.1	Patten and Mains[25]
Rhubarb juice	3.1	Patten and Mains[25]
Strawberry juice	3.4	Patten and Mains[25]
Pineapple juice	3.4–4.1	Patten and Mains[25]
Tomato juice	4.2	Patten and Mains[25]
Pear (nearly ripe)	4.2	Foà (1906)
Grape juice	4.5	Foà (1906)
"Normal plant cell sap"	5.3–5.8	Wagner (1916)
Fiscus elastica milk	5.7	Foà (1906)
Prune juice (autoclaved)	4.3	Clark and Lubs
Apple juice (autoclaved)	3.8	Clark and Lubs
Banana juice (autoclaved)	4.6	Clark and Lubs
String bean juice (autoclaved)	5.2	Clark and Lubs
Carrot juice (autoclaved)	5.2	Clark and Lubs
Cucumber juice (autoclaved)	5.1	Clark and Lubs
Beet juice (autoclaved)	6.1	Clark and Lubs
Potato juice (autoclaved)	6.1	Clark and Lubs

is it necessary, for instance, to consider the dissociation of sugars, since their acid dissociation constants are so small that these substances are generally found only in a practically molecular state in neutral or acid solutions. Likewise there are zones within which any given acidic or basic group will be found in a practically totally dissociated or practically undissociated state. Perhaps there is no more vivid way of illustrating this than by a contemplation of indicators. Above a certain zone of hydrogen ion concentration phenolphthalein solutions are colorless. Below this zone (until intense alkalinity is reached) only the colored form is observed. Within the zone the color change

garicus type to reach. This observation is of very interesting significance now that Hunter and Bushnell (1916) and Sherman (1916) have shown the dominant organisms in silage to be of the Bulgaricus type.

[25] These values were obtained by Dr. Patton and Mr. Mains of The Bureau of Chemistry. By the courtesy of Dr. Patton and Mr. Mains and of the Bureau of Chemistry we are permitted to publish these average values in advance.

with change in hydrogen ion concentration is great. The conduct of phenolphthalein, which happens to be visible because of tautomeric changes which accompany dissociation, is a prototype of the conduct of all acids. Just as we may suppress the dissociation of phenolphthalein by raising the hydrogen ion concentration of the solution, so we may suppress the dissociation of any acid. A similar illustration for the conduct of bases is obtained if we regard methyl red as a base. The general

TABLE 23

Acid agglutination optima

ORGANISM	P_H	AUTHORITY
B. paratyphi	3.7	Michaelis (1911)
B. typhi	4.4	Michaelis (1911)
Pneumococci	5.0	Gillespie (1914)
Plague bacillus	5.0	Markl (1915)

TABLE 24

Limiting reactions

ORGANISM	P_H	AUTHORITY
B. coli	5.0	Michaelis and Marcora (1912)
B. coli (low gas ratio group)	4.3–5.3	Clark (1915c)
Streptococci, Group I	4.6–4.8	Ayers (1916)
Streptococci, Group II	5.5–6.0	Ayers (1916)
Streptococcus erysipelatos	4.8	Itano (1916)
B. subtilis, acid limit	4.2	Itano (1916)
B. subtilis, alkaline limit	9.4	Itano (1916)
Yeast	2.5–2.7	Lüers (1914)
Yeast	2.3	Högglund (1915a)

relations between the strength of acids as expressed by their dissociation constants and the percentage dissociation at different hydrogen ion concentrations will be found graphically illustrated in figure 5 (Journal of Bacteriology, ii, 110).

The point we wish to emphasize is, that within certain zones of hydrogen ion concentration slight changes may have an enormous effect just as we observe them to have in the case of indicators; while in other regions no appreciable effect is observed although the hydrogen ion concentration is varied enormously.

An important instance where such a consideration is very suggestive has been noted by Michaelis (1914 b). Michaelis (1914 a) and his collaborators (e.g., Michaelis and Davidsohn (1911)) have shown that enzyme solutions conduct themselves as if the enzyme were an electrolyte. Invertase, for instance, gives an activity curve for different hydrogen ion concentrations which has the form and many of the characteristics of the dissociation residue curve of an amphoteric electrolyte. In such curves there is seldom a sharp optimum point as has sometimes been inferred, but rather an optimum *zone*. Within and beyond this zone slight changes in hydrogen ion concentration produce no great change in the activity of the enzyme, but upon the borders of the zone a small change in P_H may make a very large difference. Michaelis suggests that there may be maintained in the organs of the body hydrogen ion concentrations which lie upon the border of the zone of optimal activity of a given enzyme and that this permits the rate or the direction of enzymatic action to be controlled by slight changes in P_H.

Considerations such as these, which will become more apparent if one studies the beautiful exposition of the dissociation curves of acids, bases and amphoteric electrolytes given by Sörensen (1912) and Michaelis (1914), would lead one to believe that the methods of determining hydrogen ion concentrations should be developed to meet two general classes of problems. In the first place, when the phenomenon under investigation is connected with an electrolytic equilibrium occurring in a P_H region where the equilibrium is seriously disturbed by slight changes in P_H the method of determining P_H values should be the most reliable available. In the second case, when the equilibrium is held practically constant within a certain wide zone of hydrogen ion concentration, it will do for most purposes to determine P_H by some approximate method. Neglecting the question of availability and arguing only from the basis of scientific efficiency the electrometric method should be applied in the first case because of its greater accuracy and the colorimetric method in the second case because of its simplicity. When the nature of the process investigated is not known and we are there-

fore unable to tell *a priori* which method on the above basis should be used, then a preliminary survey may be made with the colorimetric method. This it seems to us furnishes a rational basis for judging the true usefulness of the two methods.

Exception will be taken to this point of view as a comprehensive basis since there are cases in which the electrometric method is inapplicable and the colorimetric method must be used in preference, or *vice versa*. Nevertheless, we believe that in general the true utility of the colorimetric method lies, first in its availability where approximate determinations are needed but where exact determinations are useless, and second in its value for reconnaisance.

In some instances the mere fact that a phenomenon will occur over a considerable range of P_H should be clearly distinguished from the significance of an optimum point. A case in point is the acid agglutination of bacteria. An analogy which is imperfect, but which may serve to illustrate this, is found in the conduct of indicators. Phenol red and cresol red are distinct compounds of the same series which we may compare for present purposes with two distinct strains of bacteria of the same species. The two indicators conduct themselves in a very similar way. To a casual observer their colors would appear very much alike and their color changes would appear to occur in the same range of P_H. If we introduce these indicators into standard buffer mixtures a red color will be observed with each at P_H 7.4, 7.6, 7.8, etc., just as acid agglutination of bacteria may be observed at, say P_H 4.8, 5.0, 5.2, etc. But the *half transformation point* of each indicator is *characteristic* and may be used to *identify* the compounds. Likewise it is the *optimum* agglutination point which is *characteristic* of bacteria or of their protein.

It may not be out of place to prolong this discussion, keeping in mind the general point of view we have presented, and to indicate more concretely whese the colorimetric method may be applied in bacteriology.

In regard to the "reaction" of culture media several facts have become apparent. In the first place, adjustments by the old titrimetric method gave widely divergent hydrogen ion con-

centrations in media of different composition and not very close agreement in different samples of the same medium. In spite of this, media so adjusted were successful in many cases. This would indicate that rough adjustment of the hydrogen ion concentration is all that is necessary. Such adjustments may be adequately controlled by the colorimetric method. If they are, we shall not again encounter situations like that which occurred when the composition of a standard official medium was changed without revision of the "standard degree of reaction," the P_H of the revised medium being at about the limit endured by the organism the medium was intended to nourish.

But although the rough adjustment of the P_H of culture media may serve in most cases, we should not overlook the fact that many organisms have been described as very sensitive to "reaction." As one of us pointed out in a former paper we do not know whether this sensitivity has been overestimated because buffer poor media were used or because of the inaccuracies and uncertainties of the titrimetric method, or whether it is a true sensitivity. For a reconnoisance of this problem the colorimetric method should prove valuable.

As media become simplified and approach closer to the ideal of synthetic composition they can be made up with greater assurance of a constant initial P_H. The establishment of particular reactions is especially easy when there are present phosphates or similar well defined substances which exert strong buffer action at certain zones of P_H to the advantage of bacterial growth as Sörenson has pointed out. Henderson has repeatedly emphasized the value of mixtures of basic and acid phosphates in regulating solutions at or near the neutral point, and Henderson and Webster (1907) have specifically recommended such a mixture for prolonging the activity of "lactic bacteria." It may be noted however that the *addition of mixtures* of the phosphates to media is not always necessary. Phosphates have relatively little buffer action at hydrogen ion concentrations lower than that of blood. Consequently, if a secondary phosphate is added to a medium which is relatively rich in base binding compounds, the secondary phosphate cannot greatly lower the hydrogen ion

concentration. On the other hand at and above neutrality the phosphate will exert a strong buffer action and tend to keep the medium near the neutral point. Consequently the addition of a proper quantity of K_2HPO_4 to an acid medium is an effective way in which approximately to adjust the medium to neutrality. The use of di ammonium phosphate is even safer because it cannot retain an excess of base, especially after heating, and as ordinarily obtained is a mixture of the mono with an excess of the diammonium phosphate.

Synthetic media present some special problems. Not a few of those which have been proposed have so little buffer effect that they are practically useless and although "synthetic" are physiologically variable because of the enormous difference in P_H which very slight impurities may produce. With the proper set of indicators this may be easily discovered. Media have also been proposed which, in the language of the druggist, contain "incompatibles." The original Czapek (1901) medium for molds contained magnesium and a phosphate, but the magnesium was kept from precipitation as magnesium phosphate by using an acid phosphate. Copyists have neglected to specify this and, in some cases, have even specified the use of a basic phosphate. The resulting P_H permits the precipitation of the magnesium. Doubtless there remains even in such a solution a sufficient quantity of magnesium to supply the minimum requirement, but since magnesium is one of the essential elements for mold growth it is better technique, at least, to keep in solution that which is supplied.

Certain similar aspects of synthetic media will be discussed more fully in a subsequent paper, but before leaving the topic we may call attention to the well known fact that the reaction of a medium has a considerable influence upon the decompositions which occur during sterilization. Pure glucose and pure "microcosmic salt" form an excellent medium for many purposes, but after sterilization such a solution may hardly be called a synthetic medium in the strict sense of the term. Likewise the more complex media suffer changes on sterilization which in some instances especially on overheating, are of serious impor-

tance. Among many items of special interest to media makers may be mentioned the formation of complexes between sugars and amino acids, treated by Maillard (1913), the decomposition of asparagine, an important constituent of many synthetic culture media, which has been studied by Ehrlich and Lange (1913), innumerable researches on the cleavages of sugars in alkaline solutions, and the reports of Lobry de Bruyn and Alberta van Ekenstein (1895–1900) on the transformations of sugars into one another in alkaline solutions. These last are of such grave importance to bacteriologists that most careful confirmation may be demanded. It may not be too speculative to suggest at this point that in some instances the baneful effect of overheating media may be due to the destruction of special compounds which in small quantities are essential to the best activity of fastidious organisms. It is well known that certain so-called "vitamines," which are essential to the health of higher organisms and without which disease and death ensue, are destroyed upon heating, especially when the reaction of the solution is slightly alkaline or even neutral. It is not at all impossible that analogous compounds necessary for the continued growth of highly specialized bacteria may be destroyed by sterilization at the reactions usually employed. The later work on "vitamines" recalls the earlier work on Wildier's (1901) "bios," a substance which he describes as "indispensable to the development of yeasts" (see also Amand (1903–04)). Devloo's (1906) description of this sounds like a modern paper on a vitamine. Of particular interest to the present discussion is the statement that it is stable in acid solutions but unstable in alkaline solutions (like the vitamines).

Now Michaelis and Rona (1906 b) have emphasized, more particularly with reference to the alleged decomposition of sugar at the temperature and alkalinity of the blood, that we should not transfer indiscriminately to the conditions of physiological solutions the conclusions of those who have worked with molecular concentrations of alkali or acid. The effective alkalinity or acidity as measured by the hydrogen ion concentration may be thousands of times greater in the one case than in the other.

Therefore much of the data on the decompositions which occur in bacteriological culture media and the influence thereon of "reaction" had best be studied with at least such control as the indicator method will afford.

When a correlation is observed between P_H and some effect, the mere determination of P_H alone will of course throw but little light upon the real nature of the phenomenon except in rare instances. Determination of the hydrogen ion concentration will not even distinguish whether a given effect is influenced by the hydrogen or the hydroxyl ions, nor will it always reveal whether the influence observed is direct or indirect. It is true however that even when the hydrogen ion concentration is effective through remote channels it may be very important. Therefore advantage should be taken of the comparative ease with which the concentration of hydrogen ions may be determined or controlled and their influence known or made a constant during the study of any other factor which may influence a process. From this point of view methods of determining hydrogen ion concentration take their place beside thermometers, and buffer mixtures beside thermostats. In some instances automatic regulation of a hydrogen ion concentration may be provided, to take its place beside the automatic thermo regulator. For instance, Dr. Jones of this laboratory provided a very good automatic regulation of the P_H of a medium at a desired point by keeping the medium saturated with CO_2. Since anaerobiosis was also desired the procedure in this case was one of unique beauty. For alkaline solutions a modified use of the ammonia vapor tension of ammonia solutions might do.

The influence of hydrogen ion concentrations upon solubilities has been mentioned. It extends quite naturally to many of those substances which exist in solution in aggregates which bring them within the classification of colloids. Whether by reason of its influence on dissociation or because of other phenomena the hydrogen ion concentration of a solution may influence the dispersion of colloids. Among such substances of interest to the bacteriologist are bacterial enzymes, certain toxins, and the components of bacteria and viruses which may

react as if the bacteria themselves were dispersible, and several of the membranes used for the separation of enzymes, toxins bacteria and viruses from their media. Whether certain of these enzymes toxins, etc., would have appeared in the filtrates obtained by certain workers, had the medium been given the proper reaction, it is difficult to say; but it is worth while to call attention to the remarkable observations of Maurice Holderer (1911) which have been confirmed in many details by others. He not only controlled the filterability of enzymes[27] by adjusting the reaction of their solutions with the aid of indicators, but he also obtained new enzymes from certain organisms by extractions at certain reactions. Aubel and Colin (1915) have confirmed Holderer's conclusions with some bacterial toxins.

In the study of all of these problems, in the testing of acid and alkali fermentations, in the investigation of such influences as the reaction of the medium is reported to exert on the formation of toxin by the diphtheria bacillus [see Park and Williams (1910)) and Ficker (1913)], and especially in the study of that neglected but profoundly important subject,—the relative *rates* of different bacterial enzymatic processes, the indicator method may be used with advantage, at least for reconnoissance.

There is always danger that so important a subject will be either overrated or underrated before its position has been clearly defined by extensive investigation. Let us therefore recall the view point established by Sörensen in his classic paper "Enzymstudien II." As he said, the influence of hydrogen ion concentration is analogous to that of temperature. In each case there are limits beyond which the action of enzymes practically ceases. In each case there is a broad optimal zone. In each case there is at least upon one side of this zone a region where slight changes in the condition produce great changes in the rate of action. May we not extend this analogy to bacterial activity in general, and maintain that, just as we pay attention to the temperature of the incubator, so should we pay attention

[27] Since writing the manuscript of this paper one of us (W. M. C.) has found that the passage through Berkfeld filters of the gelatine liquefying enzyme of B. proteus can be controlled by adjusting the P_H of the solution.

to the hydrogen ion concentration of the medium. For general work precise thermometry, as the physicist knows it, has no place in bacteriological technique; for general work precise hydrogen electrode measurements of P_H are unnecessary. But a consideration of the facts already established is making it evident that we must have some means of determining hydrogen ion concentration which is comparable with the use of an ordinary thermometer for determining temperature and that it is advisable to use it with the same frequency. Experience with temperature effects enables one to judge of the accuracy required in any particular case; only experience will furnish a true measure of the limitations as well as the advantages of the indicator method. Our present judgment is that certain bacteriological problems are worthy of the instrumental accuracy of the very best hydrogen electrode equipment and of the most careful and skilled technique that can be applied to that instrument, but that to discover the cases where this may be necessary as well as to deal with numerous problems where this is surely not necessary the indicator method is adequate.[28]

[28] It is perfectly obvious that the indicators and the methods which we have described in this paper with particular reference to the needs of bacteriology may prove to be useful in other fields of investigation. The excellence of some of the indicators for the determination of the P_H values of urines is shown in table 12. Dr. Gillespie (1915) has used some of the new indicators in his soil work, and Dr. Patton and Mr. Mains have used them in studies on the acidities of fruit juices. Many other special uses might be suggested.

It is perhaps less obvious that many of the considerations which have led us to abandon the titrimetric method of adjusting the reaction of culture media apply to a great many other tests. Extracts of natural products very frequently give titration curves which are similar in many respects to the curves of culture media already described. One important characteristic is the strong buffer effect exhibited in the P_H region where phenol phthalein changes. It can easily be shown that such solutions are not subject to the application of the principles which have been developed for the titration of pure solutions of strong and moderately weak acids. In the great majority of cases the titration of such solutions reduces to a method of showing mere differences in different samples without showing the analytical content of the acids or bases.

According to Trillat (1916) the ancient Romans titrated the alkalinity of natural waters with drops of red wine. Although modern standards of concentration are more exact than the wine standard used by the Romans the modern

SECTION XIX. SUMMARY

Recent developments in the study of the influence of the hydrogen ion upon various processes of physiological importance have made it evident that these studies must be extended to bacteriological problems. We have given a very brief sketch of work already accomplished in other fields and have noted some instances where the influence of hydrogen ion concentrations upon the activities of bacteria, yeasts and molds has already been demonstrated.

With due consideration for certain superiorities in the electrometric method of determining hydrogen ion concentrations we have pointed out that for general work the colorimetric method promises to be most useful.

Certain major and minor hindrances to its general application in bacteriology we have materially lessened or removed. A new series of indicators of exceptional brilliancy has been assembled from among those already described or from the several new indicators which we have synthetized for the first time. The selection is composed of the following individual compounds with which are listed the useful ranges.

choice of indicators is oftentimes no more logical than the Roman choice of the coloring matter of red wine.

The frank admission that the analytical content of acid or base in a given complex solution cannot be determined by titration to a given tint of some arbitrarily selected indicator does not necessarily destroy the empirical value of such a titration. There can be no doubt, for instance, that the titration of milk furnishes information of great value to the cheese maker or the creameryman, but it is nonsense to elaborate the simple straightforward procedure of titration so that the results even in the case of perfectly fresh milk must be stated as *percentage lactic acid!*

In the titration of the various extracts which have to be dealt with by some empirical method in food or inspection laboratories it may frequently happen that significant differences can be detected better by a simple rapid colorimetric test of the original P_H values of different samples. In other cases it may be better to titrate to some point other than the customary phenol phthalein point or rather region. In either case the brilliancy and what we now know of the reliability of the indicators we have had the pleasure of describing will, we are sure, appeal to others.

	Range P_H
Thymol blue (acid range)	1.2–2.8
Thymol blue (alkaline range)	8.0–9.6
Brom phenol blue	2.8–4.6
Methyl red	4.4–6.0
Propyl red	4.8–6.4
Brom cresol purple	5.2–6.8
Brom thymol blue	6.0–7.6
Phenol red	6.8–8.4
Cresol red	7.2–8.8
Cresol phthalein	8.2–9.8

These indicators have been tested in a wide variety of solutions such as are used for the cultivation of bacteria, and especially in colored and turbid media, and their indications compared with electrometric measurements. The correlation reveals a general agreement which may be considered satisfactory for most purposes. In certain instances discrepancies which may be attributed to "protein" or "salt" errors and in some instances discrepancies which were traced to optical effects have shown that the indicators are not always reliable for accurate measurements.

In the colorimetric determinations the Walpole compensation method for colored and turbid media has been extensively studied with the simple device of Hurwitz, Meyer and Ostenberg. The dilution method has also been elaborated. Approximate and systematized procedures have been described. Special apparatus of simple design including a special light source for use with the two dichromatic indicators have been devised.

A new set of standard comparison solutions especially designed for accuracy and ease of preparation has been described, and, as reported elsewhere, carefully studied.

Brief sketches have been given of the more important theoretical aspects of the use of indicators, and general considerations have been presented to show the proper field of usefulness of the method.

It is concluded that, with the improvements presented, the colorimetric method is available for routine as well as research purposes in bacteriology.

Applications already made have been mentioned and new uses have been suggested.

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THE USE OF A THREE PER CENT LACTOSE LITMUS AGAR PLATE FOR THE DEMONSTRATION OF B. COLI IN WATER EXAMINATION

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The use of standard lactose litmus agar plates (A. P. H. A., 1912, p. 130; A. P. H. A., 1917,) for the confirmation of the presence of *B. coli*, is complicated by many objectionable features (Frost, 1911, pp. 121, 133; Prescott and Winslow, 1915, pp. 105, 132; Rector, 1913). Organisms other than *B. coli* grow readily on this medium and are apt to spread over the surface of the plate. This spreading either prevents the growth of the colon bacilli or masks their typical reaction and often renders the fishing of colonies for further identification either difficult or impossible. Also, as a rule, in order to insure proper distribution of colonies, two or more plates must be made for each tube to be confirmed.

Streak plates made upon Endo's fuchsin-sulfite medium have been used in many laboratories to offset these difficulties. Endo's medium is made with a 3 per cent agar base (A. P. H. A., 1912, p. 133). Plates are poured and allowed to harden thoroughly and inoculation is made by streaking out a loop of the liquid medium to be confirmed, onto the surface. After a little experience in their use, good distribution of colonies on these plates may be secured, using only one plate for each tube. Endo's medium plates are very restrictive in action and this fact has been ascribed to the fuchsin and sulfite. The results obtained by the use of the medium described in this paper would however, seem to indicate that most, if not all, of the restrictive action is due to the high concentration of agar used.

In spite of the advantages of Endo's medium, lactose litmus agar plates are preferred by many water-workers (Frost, 1911, pp. 121, 133; McLaughlin, 1913, p. 12), and it is the confirmatory medium in most common use. Frost has shown that the ordinary (1 per cent agar-poured) lactose litmus agar plate and the Endo's medium plate are equally diagnostic as to confirmation of presumptive tests (Frost, 1911, pp. 118, 121). Choice should therefore be based on considerations of convenience.

In an attempt to eliminate the difficulties incident to the use of lactose litmus agar, this medium was made up using the regular 3 per cent agar "base" as made for Endo's medium, prepared with the 1 per cent peptone and 1 per cent meat extract. As used by the writer, the base was put up in 400 cc. portions, to which were added when ready for use, 16 cc. of a sterile 25 per cent lactose solution, and 12 cc. of a sterile 8 per cent litmus solution. This amount of medium was used to pour 25 to 30 plates, which were allowed to harden open to the air. Plates not immediately used were stored at ice-chest temperature, without any apparent change in appearance or results. This medium seems just as restrictive to air forms as Endo's medium. Five or six plates, made as above and subject to normal air contamination during thirty minutes of hardening, showed no growths after fourteen days storage in the refrigerator.

This method of making lactose litmus agar plates could not, of course, be used for direct plating of water or sewage samples for a "red colony" count, without much difficulty in manipulation (Frost, 1911, p. 121). In general, however, the direct red colony count has been definitely discarded in favor of the dilution method, for quantitative determinations of *B. coli*. Therefore, the only use for lactose litmus agar is as a confirmatory medium.

To check this new medium against Endo's medium, fermentation tubes showing gas were inoculated simultaneously onto the surface of plates of both media. When typical colonies appeared on both plates, the demonstration was carried no further. When the colonies on either or both media were atypical, one or more were fished from each plate. These were inoculated into lactose bouillon tubes and onto agar slants. If more than 10

per cent gas was formed within forty-eight hours and the slant showed only Gram negative non-sporeforming bacilli, the colonies were considered *B. coli*. It was found that in all cases where the tubes showed gas, this was due to Gram negative, non-sporeforming organisms.

Summary of results of comparison of 3 per cent agar lactose litmus plates with Endo's medium plates

	NUMBER OF TUBES PLATED	NUMBER SHOWING B. COLI	
		Lactose litmus agar 3%	Endo's medium
Plates typical with both	101	101[1]	101[1]
Plates atypical with both	65	5[2]	13[2]
Typical lactose litmus agar, atypical Endo	2	1[2]	1[2]
Atypical lactose litmus agar, typical Endo	24	6[2]	13[2]
Total	192	113 (59%)	128 (66.6%)

[1] Considered *B. coli* without further confirmation.

[2] Demonstrated as *B. coli* Gram-negative, non-spore-forming bacilli, giving gas from lactose.

Note must be made of the fact that the low percentages of confirmations, 59 per cent for the lactose litmus agar and 66 per cent for the Endo's, were due to the fact that only tubes originally showing less than 10 per cent of gas in twenty-four hours were used for this work. The average confirmation for all tubes, for this laboratory, for the year 1914, was about 92 per cent.

SUMMARY

Lactose litmus agar plates prepared with 3 per cent agar and inoculated by streaking onto the surface are free from many objections incident to standard lactose litmus agar plates. They are more convenient, restrict entirely any spreading forms, and give results approximately equivalent to Endo's medium plates. The latter, however, appears in the writer's estimation to be somewhat preferable, as giving slightly higher results.

The writer wishes to acknowledge the kind suggestions of Dr. W. H. Frost in the preparation of this paper.

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A SIMPLE METHOD FOR STAINING THE CAPSULES OF BACTERIA

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The possession of mucinous envelopes by certain bacteria, apart from their purely morphological interest, has a great value in the identification of such organisms and many methods have been devised for their demonstration. The number of such methods however argues against any one being completely satisfactory.

The introduction of a new technique into this already overcrowded field can be justified only if it offers distinct advantages over any or all of the older methods. The staining of capsules as a rule, particularly in inexperienced hands, is time consuming and attended by many failures, and a new method to be acceptable should as far as possible eliminate those factors which experience has shown to be the most general cause of failure.

These factors may be the uncertain or variable quality of the diluent employed, the impossibility of giving absolute directions as to the amount of heat required in fixation or in staining, or the use of fixing agents, such as strong chemicals, that in themselves tend to shrink the capsule.

The search for a method that would overcome these difficulties led to the employment of an artificial menstruum prepared from Nutrose[1] (as directed below), the abandonment of heat as a fixing agent and as an aid in staining, and the use of weak solu-

[1] Nutrose (sodium caseinate) is a proprietary food preparation made in Germany and, as under present conditions it may be unobtainable, a fairly efficient substitute for the 3 per cent Nutrose solution may be made from milk by the following procedure: Render milk as nearly fat free as possible by means of centrifugation, add 1 per cent of 2 N sodium hydrate and bring to a boil, after cooling add ether and shake. After a few minutes decant off the ether. The remaining opalescent fluid can then be employed in place of the Nutrose solution.

tions of lactic acid as precipitating agents. Thin films made from an emulsion of organisms in 3 per cent Nutrose solution are precipitated immediately by dilutions of lactic acid of a strength of 0.1 of 1 per cent causing a perfect fixation of the organisms and capsules.

Experience has shown that 0.25 to 0.5 of 1 per cent of lactic acid gives the best results, that it is perfectly feasible to introduce the dye employed directly into the fixing solution, thus simplifying the technique, and that the addition of acetic acid in a strength of 1–10,000 improves the definition.

Method of preparing the reagents employed

Solution 1. To be used as a diluent.

Three grams of Nutrose are sifted into 100 cc. of distilled water and heated to 100°C. in the Arnold sterilizer for one hour.

Add 5 cc. of 2 per cent aqueous solution of carbolic acid to serve as a preservative. Decant into test tubes and allow to settle.

Employ the supernatant fluid as the diluent.

(Since the supernatant fluid tends to become thinner by constant precipitation of the Nutrose the solution should occasionally be reboiled.)

Solution 2. Fixing and staining solution.

2 per cent aqueous solution of carbolic acid, 100 cc.
Concentrated lactic acid, 0.25–0.5 cc.
1 per cent acetic acid, 1 cc.
Saturated alcoholic solution of basic fuchsin, 1 cc.
Carbol-fuchsin (old), 1 cc.

(This solution must be kept tightly corked, and then keeps very well.)

Experience has shown that in the above solution the addition of old and fresh fuchsin as given makes a better product than an increase in the amount of either one alone.

Technique of staining

1. Employ the Nutrose solution (no. 1) as a diluent, emulsifying the bacteria in one or two loopfuls and then spreading in as thin a film as possible with the loop. The use of the edge of a slide in spreading the film, as in blood work, is not to be recommended.
2. Allow to dry in air.
3. Cover the film with the fixative and staining solution (no. 2) and allow to act for thirty to forty-five seconds.
4. Wash quickly in water, dry and examine.

These steps take about one and half minutes.

In examining the films the best pictures are usually found in the thinnest portions near the edges.

The use of fuchsin as a dye has proven to be generally satisfactory for a routine stain but any of the more diffuse basic dyes may be employed. Methyl violet added in 1 per cent of a saturated alcoholic solution gives probably the best pictures due to the failure of the Nutrose film to stain, the organisms appearing as blue black bodies surrounded by delicate violet capsules. This dye, however, tends to precipitate out of the mixture in a few days and the preparations are apt to fade.

The above method has been in use in this laboratory for three years and has been used in the class room during this period with very satisfactory results, being taught in conjunction with one of the older methods so that a comparison of results on a very large number of preparations was possible. It rapidly became the method of choice by students due to the simplicity of the technique and the very high percentage of successful results secured. The results of preparations made by this method are shown in figures 1 to 6.

Since the presence of a demonstrable capsule has such differential value, a method of this kind to be acceptable must fail to show such a structure on those organisms which are commonly held to be non-capsulated.

In the course of an investigation along another line this method has been tried on streptococci, staphylococci, members of the Gram negative coccus group, and many flagellate bacteria and has in no instance shown a capsule on these organisms although a similar structure may be demonstrated on all by means of a special technique which will be the subject of a subsequent report.

The advantages claimed for the above method of capsule staining are:

1. Standardization of the reagents employed.
2. The elimination of personal judgment as a factor by precise instructions as to the various steps.
3. Simplicity of technique.
4. Reliability of results.

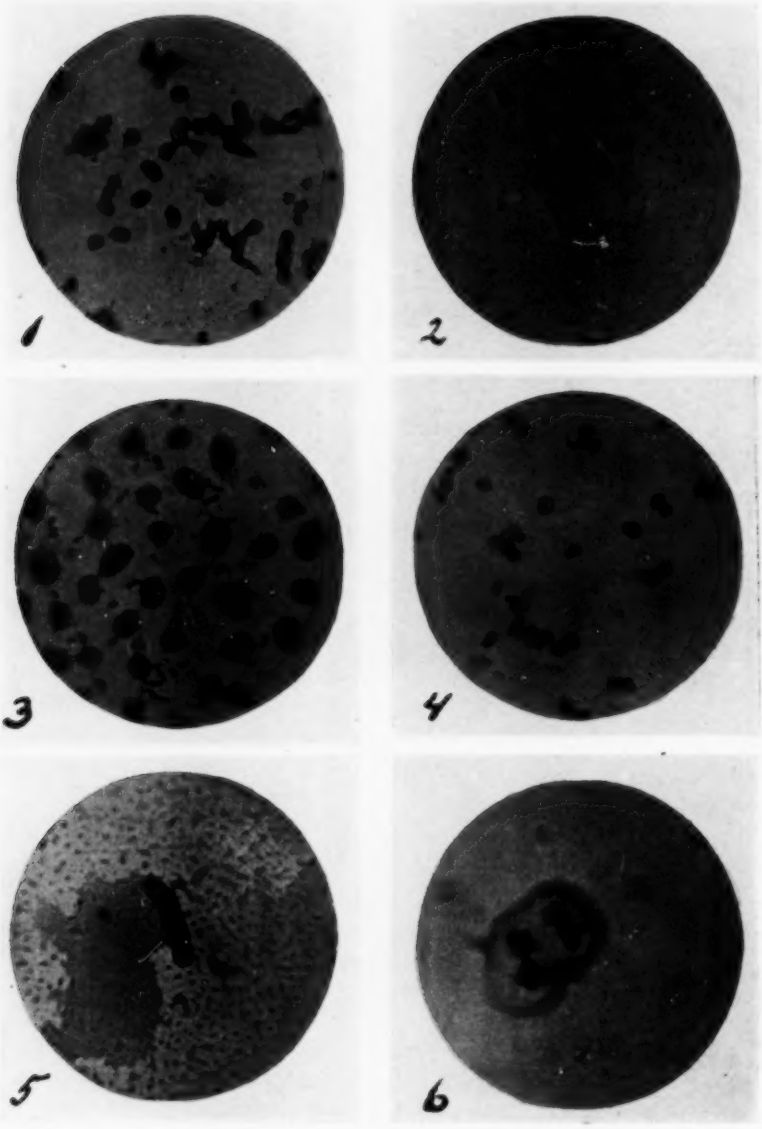

FIG. 1. PNEUMOCOCCUS
FIG. 2. STREPTOCOCCUS MUCOSUS
FIG. 3. PNEUMOBACILLUS OF FRIEDLÄNDER AND BACILLUS PYOCYANEUS
FIG. 4. BACILLUS AEROGENES
FIG. 5. BACILLUS AEROGENES
FIG. 6. MICROCOCCUS TETRAGENUS SHOWING THE INDEFINITE OUTLINES OF THE GELATINOUS ENVELOPE

A CURIOUS ACCIDENT DUE TO B. PNEUMONIAE

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In the early summer of 1916 three barrels of soft soap were bought for use in scrubbing floors at the Ambulance de l'Ocean, La Panne, Belgium. These were placed in the storeroom of the Ambulance. About two weeks later one of the barrels exploded and its foaming contents ran out on the floor. During the next two or three days the other two barrels burst and the violently fermenting soap "boiled" out over the floor of the storeroom.

Bacteriological examinations were made of samples of the frothy soap from each barrel. Smears, made with some difficulty, and stained, showed great numbers of Gram negative bacilli approximately 0.8μ by 3 to 7μ. A few longer forms were also seen. Most of the bacilli occurred singly or in pairs, but chains of 6 to 20 elements were not infrequent. No spores were found.

Milk inoculated from the soap and heated to 80°C. for fifteen minutes remained sterile. Milk similarly inoculated, but not heated, showed in twenty-four hours coagulation with formation of a small amount of gas, but without any digestion of the curd.

On plain agar plates the colonies were practically all of one kind, round, 1 to 2 mm. in diameter, moist, elevated, semi-opaque, with regular edges. The growth strung away from the needle point when the colonies were fished.

Stab cultures were made from three similar colonies into tubes of glucose agar. The following morning the cotton plugs and part of the medium were found on the floor of the incubator, so violent had been the fermentation. Even plain agar slants inoculated at the same time were pushed up from the bottom of

the tube by the development of gas from the growth of bacilli in the water of condensation between the medium and the walls of the tube.

Three strains, designated "A," "B" and "C," were isolated. They showed some minor cultural differences, but were quite similar morphologically. The bacilli measured approximately 0.5 to 0.9μ in thickness by 2 to 6μ in length. In glucose broth strain "A" showed a tendency to grow in chains; but in most media all of the strains grew singly or in pairs. They were Gram negative. In milk cultures there was a more or less definite capsule. The organisms were non-motile.

On agar slants strain "A" produced a very vigorous, elevated, gray, translucent, spreading growth with regular edges. Smears from these cultures showed bacilli similar in all respects to those seen in stained preparations from the fermenting soap.

In plain broth there was uniform turbidity. The medium within forty-eight hours became very viscid and mucus-like, of that there was no tendency to sedimentation even after four weeks. There was a very slight annular scum.

Milk was rendered acid, a soft curd was formed and a few bubbles of gas were produced. There was no digestion of the coagulum.

The bacilli grew well in gelatin without liquefying it. A characteristic "nail-head" growth was not observed, although the growth on the surface was quite vigorous.

This strain fermented glucose, glycerine and mannite violently producing approximately 100 per cent of gas in a fermentation tube in twenty-four hours. In sucrose broth 80 to 90 per cent of gas was produced. Lactose was attacked less vigorously, but gas was formed to the extent of 50 to 60 per cent. Starch was not fermented.

In glucose broth this strain gave a Voges-Proskauer reaction. This reaction was not observed in lactose, sucrose, mannite or glycerine broths.

Strain "B" differed from the above in that it did not ferment lactose, did not coagulate milk, produced only 20 to 30 per cent of gas in sucrose broth and did not give the Voges-Proskauer

reaction. The viscidity of broth cultures was a little less pronounced. Strain "C" produced about 50 per cent gas in lactose broth but did not always coagulate milk, and did not give the Voges-Proskauer reaction. Otherwise it was similar to strain "A."

It is evident that of the three stains, "A" and "C" fall into Perkins'[1] Group I which ferments all sugars, while "B" belongs to Group II which ferments all sugars except lactose. Only one of the organisms (Cleveland 11) studied by Perkins fermented glycerine as vigorously as the three strains isolated from this explosive soap.

The chief interest in the organisms here described lies in the fact that they grew vigorously in a rather strongly alkaline cheap soap containing very little protein matter; that they attacked glycerine with remarkable avidity; and that the three strains, although isolated from the same source and belonging to the mucosus-capsulatus group of bacilli, showed slight cultural differences among themselves.

[1] Jour. Infect. Dis., 1904, **1**, 241.

THE FALLACY OF REFINED READINGS OF GAS PERCENTAGES IN THE FERMENTATION OF LACTOSE PEPTONE BILE AND LACTOSE BROTH

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The detection of the *B. coli* group in water, milk, oysters, and sewage effluents is of the utmost importance to the sanitary bacteriologist, for knowing its normal habitat to be the intestine of man and the higher animals, he at once infers that its presence must indicate pollution of intestinal origin. It is now firmly established that the *B. coli* group is an index of fecal pollution. Immediately after its discovery by Escherich in 1885, workers in this field of science began to devise methods of identifying the colon organism; as a result of their investigations we have various tests proposed and used quite extensively down to 1906, which aimed primarily at determining the presence or absence of the *Bacillus coli* group. The following tests were used:

A. Litmus lactose agar plate method (Wurtz, 1892)

This test is based on the principle that *B. coli* has the property of fermenting lactose with the production of acid. *B. coli* may be identified by the red colonies on the blue litmus lactose agar plates. The chief objections to this method are these: (1) Other microörganisms, notably the streptococci, also produce red colonies on litmus lactose agar plates. (2) Too much time is taken up in making supplementary tests for further identification.

B. Glucose broth-enrichment test (1901)

This test depends upon the fact that *B. coli* in glucose broth produces gas, which can be measured. In the early years of water bacteriology, this test was merely the first step in a long scheme of analysis; experience soon showed that 25 per cent or more of gas in the fermentation tube or in the small inverted vial was sufficient to justify the presumption of the presence of *B. coli*, and it now took the name "presumptive test." The chief advantage of this test is that only twenty-four to forty-eight hours are required to determine the presence of *B. coli*. The great disadvantage, however, is that it sometimes fails to show the presence of the colon group, since the culture medium is particularly suited to the development of other bacteria, which inhibit the colon group. Furthermore, it has been found that the factors of temperature, storage and reaction of the medium have much to do with this test.

C. Phenol broth (Reynolds, 1902)

The problem of the struggle for supremacy in the glucose broth tube was soon solved. By the addition of a small amount of a weak solution of phenol the ordinary water bacteria could be inhibited in growth to the advantage of the colon bacilli. Again, however, disadvantages arose even more serious than those affecting the pure glucose tube, since, in waters of fairly pure quality, phenol inhibits the growth of the *B. coli* group.

D. Eijkman test (1904)

Eijkman suggested that water forms might be eliminated in this test if the temperature of incubation was made 46°C. It was soon pointed out that weak strains of colon bacilli are also inhibited at that temperature.

E. Neutral red reaction (Rothberger, 1898)

This test depends on a color reaction. Objections have been made to its use because of the relatively large number of organisms unrelated to the colon group giving this reaction.

F. Lactose broth test

Experimental evidence is not lacking to show the usefulness of lactose broth as a medium for making the presumptive test for *B. coli*.

It has the decided advantage over the glucose broth medium that most water bacteria are inhibited to the advantage of the colon group; the fact to be emphasized here is that both these tests depend on the fermentation of sugars with the production of acid and gas.

G. Lactose bile test (Jackson, 1906)

It was not until 1906 that Jackson, working on this problem, suggested the use of a fermentation test such as had been used before in the case of the glucose and lactose broths but substituted, for these media, lactose bile. He believed that bile would most effectively represent conditions as the colon bacilli find them in the intestinal tract. Experiments soon proved the value of this test because: (1) The test can be performed in twenty-four to forty-eight hours. (2) Bile salts inhibit the ordinary water forms, and at the same time offer an ideal culture medium for typical *B. coli* (selective action). (3) Bile salts inhibit the growth of the glucose positive, lactose-negative fermenting organisms.

Of all these tests which have been offered, the lactose broth presumptive test and the lactose bile presumptive test have been used most extensively in the last decade. Against the test as a means of identification of the colon group the writers have no protest, but in its application in the laboratory, and in its interpretation, a change seems necessary.

It is a common practice in laboratories to read gas production in fermentation tubes and inverted vials very accurately, sometimes to the fractional part of a unit, thus: $33\frac{1}{3}$ per cent gas = + *B. coli*, $66\frac{2}{3}$ per cent gas = + *B. coli*, 75 per cent gas = + *B. coli*, etc. Of what value are these numbers, expecially fractions to the left of the equal sign to the practical laboratory man? He is primarily interested in what follows the equal sign. It is the purpose of this paper to show that the percentages of gas produced in lactose peptone bile and lactose broth have little quantitative value, and that there can be only one accurate way of reading and recording gas in these media, and that is, by positive (+) and negative (−) signs respectively. Among the factors influencing the percentage of gas production we have found: (1) Temperature, (2) time, (3) initial re-

action of the medium, (4) length of the inverted vial, (5) source of bile, (6) absorption of formed gas.

The changes and variations produced by these factors are so well-defined, as will become apparent to the reader, that refined gas readings are absolutely valueless in performing these presumptive tests for the colon group and results could be expressed more scientifically by the plus (+) and minus (−) signs. Our investigation was begun with the aim of studying only the lactose peptone bile test, but toward the end of the experiments it was deemed best to show that the same factors influence the readings in the lactose broth test, which is now being used in many laboratories. (Lactose peptone bile gives a precipitate of acid protein, which makes gas reading very difficult or impossible, and for this reason it is being dropped in making water analyses; lactose broth is taking its place.)

CULTURES USED IN THE INVESTIGATION

1. B. coli. Members of the *B. coli* group were isolated from Hudson River water, and cultures were kept in stock in the laboratory. The following characteristics are assigned to this organism: (1) short bacillus with rounded ends, (2) Gram negative, (3) non-liquefaction of gelatin in sixteen days, (4) grayish white growth on agar 20° C., (5) facultative anaerobe, (6) non spore-forming, (7) gas in lactose peptone bile, (8) fermentation of glucose and lactose with the production of acid and gas.

2. Hudson River water (taken from the river as it was needed in the experiments—from January 25 to June 10, 1916). Along with pure cultures of *B. coli*, Hudson River water was used, the purpose being to compare the action of pure and mixed cultures. Throughout the experiments, reference will be made to *pure* and *river* cultures. Various dilutions were used expressed as follows: 0 = 1 cc., 2 = 0.01 cc., 4 = 0.0001 cc. dilution.

METHODS

The culture media were prepared according to the Standard Methods of Water Analysis of the Laboratory Section of the

American Public Health Association (1912). The reaction, unless otherwise stated, was neutral to phenolphthalein. The greatest care was taken to have all the tubes uniform in size as well as in the amount of medium each contained. All were sterilized in the same manner, using 15 pounds steam for fifteen minutes.

METHODS OF DETERMINING THE AMOUNT OF GAS PRODUCED (PER CENT)

Vials of uniform size (2 inches long) were used in the greater part of the work. In accordance with the custom of reading gas percentages, a vial filled completely with gas, was read 100 per cent, and other readings made in proportion to the amount of gas in the inverted vial. In order to facilitate reading and at the same time to obtain a more accurate reading, a scale was constructed on a small card, which showed all the necessary subdivisions to enable the writers to read the percentages accurately; the card was held against the tube and the reading made.

RELATION OF TEMPERATURE TO THE AMOUNT OF GAS PRODUCED

Fifty 1 cc. portions of the 0, 2, and 4 dilutions of Hudson River water were inoculated into lactose peptone tubes. Twenty-five 1 cc. portions of a twenty-four hour nutrient broth of *B. coli* (0, 2, 4 dilutions) were also inoculated into another series of tubes. Four such sets of tubes were inoculated, making the total 900 tubes, and 225 were subjected to each temperature, there being four temperatures studied: 31°C. incubator temperature, 35°C. incubator temperature, 37°C. incubator temperature, 39°C. incubator temperature.

At the end of the twenty-fourth hour, the tubes were removed from the incubator and the percentages of gas recorded.

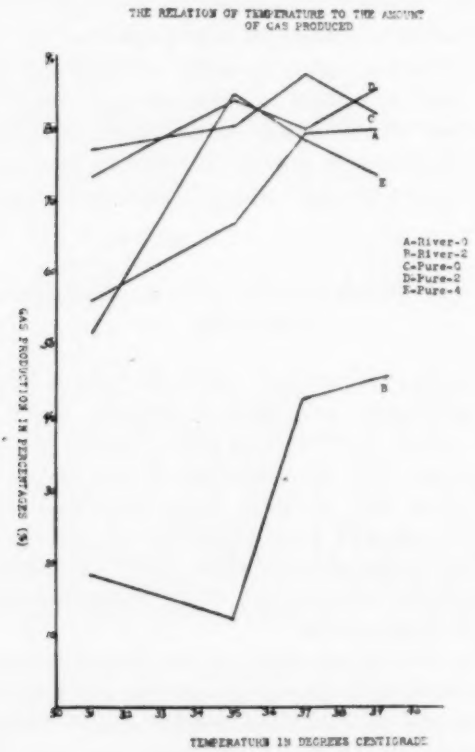
Reference to the plot of these results clearly brings out the variations caused by the temperature factor. The reader will

TABLE 1

Amount of gas produced at different temperatures by members of the B. coli group in pure and mixed cultures

TEMPERATURE	RIVER			PURE		
	0	2	4	0	2	4
°C.						
31	56.2% (20–95)	17.9% (0–55)	0%	77.8% (50–90)	73.0% (55–85)	51.6% (15–80)
35	67.5% (30–90)	12.0% (0–100)	0%	80.2% (65–90)	83.6% (70–90)	84.2% (60–90)
37	79.0% (35–100)	42.5% (0–90)	0%	87.5% (65–100)	79.3% (95–100)	77.9% (65–95)
39	79.9% (15–100)	45.6% (0–95)	0%	81.8% (65–100)	86.2% (75–100)	73.3% (60–90)

The results are the average of 50 readings in the case of the river cultures, and the average of 25 readings in the case of the pure cultures.

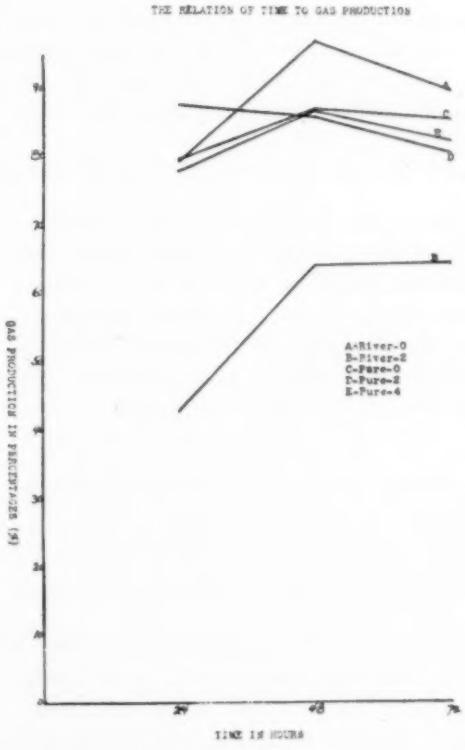


PLOT 1

note the results under river 2, which are specially significant in that they show the widest range in gas readings when the same media and cultures were used.

RELATION OF TIME TO THE AMOUNT OF GAS PRODUCED

In this experiment, one cubic centimeter portions of the various dilutions of the pure and river cultures of the *B. coli* group were inoculated into lactose peptone bile tubes. Gas readings were made at the end of twenty-four, forty-eight and seventy-two hours. Incubation temperature, 37°C.



PLOT 2

TABLE 2

Amount of gas produced in different periods of time, by the B. coli group in pure and mixed cultures

HOURS	RIVER			PURE		
	0	2	4	0	2	4
24	79.0% (35–100)	42.5% (15–90)	0%	87.5% (65–100)	79.3% (45–100)	77.9% (65–95)
48	97.1% (50–100)	63.9% (0–100)	0%	86.0% (60–100)	87.0% (70–100)	86.9% (75–100)
72	89.9% (75–100)	64.3% (0–100)	0%	80.6% (65–100)	85.5% (65–100)	82.3% (70–100)

River culture results are the average of 50 tubes. Pure culture results are the average of 25 tubes.

It will be noted that the members of the *B. coli* group produce the maximum amount of gas at the end of the forty-eighth hour. The results show an absorption of gas beginning at the end of the forty-eighth hour. It will be seen that gas production is a variable, depending upon the factor of time.

THE RELATION OF THE LENGTH OF VIAL TO GAS PRODUCTION

The purpose of this experiment was to determine the difference in gas production (gas readings) when vials of different lengths were used. Vials from 1 inch to 5½ inches in length were used, and the same amount of culture medium was added to each tube no matter what vial was placed in the tube. All the tubes were inoculated with 1 cc. of Hudson River water and incubated at 37°C. for twenty-four hours.

TABLE 3

Amount of gas produced in vials of different lengths

Vial length, in inches..	1	1.5	2	2.25	3	3.5	4	4.5	5	5.5
Readings in per cent...	95	70	61	62	51	43	58	61	70	41

The results in each case represent the average of five tubes.

The results should not be surprising; such have been the comparative results obtained continually in sanitary bacteriology. What we have done has been to choose arbitrarily a 2 inch vial to measure gas production—but is it not clear and evident that the length of the vial is an important factor in the end result?

RELATION OF THE INITIAL REACTION OF THE MEDIUM TO THE AMOUNT OF GAS PRODUCED

Tubes of lactose peptone bile and lactose broth, to which had been added varying amounts of acid and alkali were inoculated with 1 cc. of a twenty-hour nutrient broth culture of *B. coli* and 1 cc. of Hudson River water, and incubated at 37°C. Readings were made at the end of twenty-four and forty-eight hours.

TABLE 4

Amount of gas produced in lactose peptone bile, with varying initial reactions, by B. coli, in pure and mixed cultures

REACTION	RIVER		PURE	
	24 hours	48 hours	24 hours	48 hours
+1%	55.9% (35–90)	71.1% (60–80)	99.2% (60–100)	81.8% (70–95)
+2%	39.4% (5–80)	55.8% (30–75)	94.9% (30–100)	74.0% (65–90)
+3%	34.2% (5–70)	34.7% (5–60)	95.2% (50–100)	68.0% (55–80)

Results are the average of 35 readings.

TABLE 5

Amount of gas produced in lactose broth, with varying initial reactions, by B. coli, in pure and mixed cultures

REACTION	RIVER		PURE	
	24 hours	48 hours	24 hours	48 hours
+2.0%	0.0%	15.0% (10–20)	0.0%	0.0%
+1.5%	10.0% (5–20)	21.4% (10–30)	5.0% (5–10)	11.4% (10)
+1.0%	19.3% (15–35)	24.3% (20–45)	17.9% (10–30)	20.0% (15–30)
+0.5%	15.7% (10–20)	23.6% (20–35)	22.1% (20–30)	24.3% (20–35)
0.0%	20.7% (10–25)	29.9% (20–40)	29.2% (25–35)	30.8% (25–40)
−0.5%	21.4% (15–30)	29.3% (25–30)	27.5% (20–30)	33.3% (30–40)
−1.0%	23.3% (15–30)	32.5% (20–45)	35.8% (30–40)	46.6% (40–50)
−1.5%	16.4% (15–25)	23.6% (20–30)	32.7% (25–40)	45.6% (40–55)
−2.0%	11.4% (5–20)	39.2% (30–60)	22.9% (10–30)	55.8% (50–70)

These results are the average of 6 readings.

INDIVIDUAL VARIATION

The next point taken into consideration was individual variation. The best way to show that gas readings are fallacious

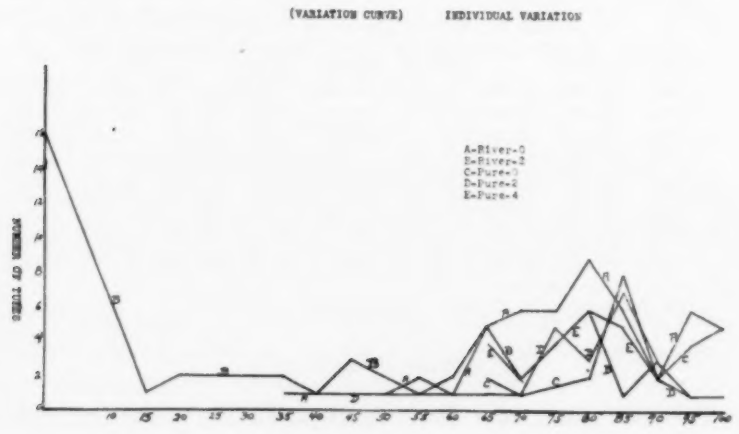
and have no real quantitative value is to present the variations not caused by any particular constant factor, but rather variations within a given group under similar conditions. Tubes of lactose peptone bile and lactose broth were inoculated with

TABLE 6

Individual variation within the group (variation curve)
Bile medium

		PER CENT OF GAS																			
		0	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
River	0							1			1	2	1	5	6	6	9	6	2	6	5
	2	16		1	2			2	1	3		1	2	5	2	4	6	1	3		
Pure	0													2	1		2	8	2	4	5
	2									1	1			1	1	5	3	7	3	1	1
	4													4	2	4	6	5	2	1	
Lactose broth, medium																					
River	0		2	3	13	7	2														
	2		1	3	12	4	1	1	1												
Pure	0					10	10	4	1												
	2				1	3	14	3	2	2											
	4				3	6	4	3	4												

Temperature, 37° C.



PLOT 3

the pure and river cultures (various dilutions) and incubated at 37°C. for twenty-four hours, after which readings were made.

Reference to the table and to the plot showing the variation curve will indicate the wide range in the amount of gas produced. These results, perhaps give the most convincing evidence as to the utter uselessness of gas readings as quantitative values, since the same substance inoculated with the same organism gives such a wide variation.

RELATION OF THE SOURCE OF BILE TO GAS PRODUCTION

It was believed that bile from different cows might produce variations in gas production, and therefore, culture media were prepared using the bile from ten different cows. The culture tubes were inoculated with the pure and river cultures of the colon bacillus, and readings made at the end of twenty-four and forty-eight hours.

As soon as the bile was received from the slaughter-house, the following characteristics were noted: (1) acidity, (2) color, (3) consistency.

TABLE 7

BILE NUMBER	CONSISTENCY	COLOR	REACTION
			per cent
1	Clear, translucent	Light green	+0.5
2	Clear, translucent	Light green	+0.7
3	Heavy liquid, opaque	Green—black	+0.8
4	Slimy, slight yellow precipitate, opaque	Black	+0.5
5	Clear, translucent	Dark green	+——
6	Heavy liquid, green precipitate	Dark green	+0.8
7	Very heavy yellow precipitate	Dark green	+0.8
8	Slight precipitate	Dark green	+0.5
9	Slight cloudy precipitate	Dark green	+0.55
10	Clear liquid	Green—black	+0.55

TABLE 8

Amount of gas produced in the various biles

BILE NUMBER	RIVER	PURE	RIVER	PURE
	Twenty-four hours		Forty-eight hours	
1	39.0% (20–65)	63.5% (50–70)	Readings could	72.0% (60–80)
2	70.6% (60–80)	81.0% (75–90)	not be made	85.0% (75–90)
3	53.0% (5–85)	72.0% (55–75)	on account of	82.0% (75–90)
4	40.0% (20–65)	69.0% (35–80)	a heavy yel-	81.0% (75–85)
5	29.0% (15–45)	65.0% (60–75)	low precipi-	72.0% (65–75)
6	40.3% (10–75)	84.0% (75–100)	tate of acid	94.0% (80–100)
7	42.5% (10–75)	82.5% (80–85)	protein.	86.0% (80–90)
8	25.6% (10–45)	48.0% (40–65)		73.0% (65–80)
9	29.4% (5–45)	68.0% (65–70)		79.0% (75–85)
10	21.3% (5–45)	42.5% (40–50)		63.0% (60–70)

CONCLUSIONS

From the data presented in this paper, it will be seen that:

1. The following factors cause variations in the gas readings in lactose peptone bile and lactose broth.

a. Temperature.

b. Time of incubation.

c. Initial reaction of the culture medium.

d. Length of inverted vial.

e. Source of bile.

f. Absorption of formed gas.

2. The gas readings in a given group vary within a wide range.

Since all these factors play an important part in the final gas readings, which necessarily must vary considerably, and since no real quantitative value can be assigned to any observation of gas production, under such conditions it seems that refined readings of gas percentages have no scientific justification in sanitary bacteriological analyses and that gas production could be recorded more conveniently and scientifically by positive (+) and negative (−) signs respectively.

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NOTE ON THE REACTION OF THE PARATYPHOIDS, ALPHA AND BETA, IN LITMUS MILK[1]

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Aside from the agglutination test, the most common method of differentiating between the paratyphoids, alpha and beta, is the reaction with litmus milk. Alpha is usually stated to give a permanent acidity while beta is said to give a permanent alkaline reaction after an initial acidity. Lehmann and Neumann (1912), for example, state that for *B. paratyphosus A*, litmus milk "bleibt sauer (*B. paratyphosus B* wird alkalish und blau.)" It is stated, moreover, that in litmus milk alpha is, "wie Typhus, ohne Aufhellung." While engaged in a broader study of the relationships of pathogenic milk-alkaline organisms as a whole, the attention of the writer was engaged by reactions of the paratyphoids which appeared to depart from the usually accepted type.

The data to which attention is directed are presented in the accompanying table, which shows the reaction of eight strains of *B. paratyphosus A*, five strains of *B. paratyphosus B*, three strains of *B. enteritidis*, two strains of *B. suipestifer*, five strains of *Bact. pullorum*, and (for controls giving permanent neutral reaction to the time of evaporation) three strains of *B. avisepticus*.

Except for Cultures 5 and 8, the strains of *B. paratyphosus A* were received from Dr. Krumwiede of the New York City Department of Health. They were checked by agglutination tests and were found to correspond with other strains of *B. paratyphosus A*. Similar agglutination tests showed the approxi-

[1] Contribution 230 from the Agricultural Experiment Station of the Rhode Island State College.

TABLE 1

*Showing the reaction of certain strains of B. paratyphosus alpha and beta, and of some other bacteria, in litmus milk**

CULTURES	REACTION AS OBSERVED AT INTERVALS (DAYS) AFTER INOCULATION OF MILK TUBES															TRANSLUCENCY
	2	4	6	8	10	12	14	18	22	34	38	50	59	90	133	192	
B. paratyphosus α																	
5	+		+		+		+	+	N		N	N		− − −			−
8	N		N		+		+	+	N		−	−		− − −			−
24	+		+		+		+	+	N		N	N		−			−
27	L	+	+		+		+	+	N		−	− −		− − −			−
28	L	+	+		+		+	+	N		N	N		− − −			−
29	L	L	+		+		+	+	N		N	−		− − −			−
36	L	L	+		+		+	+	N		N	N		− − −			−
37	L	L	+		+		+	+	N		N	N		− − −			−
B. paratyphosus β																	
6	L		L	−					− − −	− −				− − −			+
9	L		L	−					− − −	− −				− − −			+
26	L		L	−					− − −	− −				− − −			+
32	L		L	−					− − −	− −				− − −			+
39	L		−	−					− − −	− −				− − −			+
B. suipestifer																	
33	L	L	L	−	−		−	−	− −			− −		− − −			+
98	L	L	L	−	−		−	−	− −			− −		− − −			+
B. enteritidis																	
1	PR		CR				−	−	− −		− −			− − −			+
25	PR		PR				−	−	− −		− −			− − −			+
31	L		CR				−	−	− −		− −			− − −			+

TABLE 1—Continued

CULTURES	REACTION AS OBSERVED AT INTERVALS (DAYS) AFTER INOCULATION OF MILK TUBES															TRANSLUCENCY
	2	4	6	8	10	12	14	18	22	34	38	50	59	90	133	192	
Bact. pullorum																	
34	L	+	L	L			L	L	N	−	−				− −		+
											−				−		
56	L	+	L	L			L	N		N	−		− −	−	− −		+
													−	−	− −		
59	L	+	L	L			L	N		N	−					− −	+
																− −	
89	L	+	L	L			L		N		N	−		−		− −	+
														−		− −	
93	N	+	L	L			L		N			N		−	− −		+
														−	−		
B. avisepticus																	
48	N		N				N	N	N		N	N	N	N		N	−
52	N		N				N	N	N		N	N	N	N		N	−
83	N		N				N	N	N		N	N	N	N		N	−

* N = neutral; L = light; (+) = acid; (−) = alkaline. The degree of reaction is indicated by the number of signs.

mate unity of the paratyphoid B group, the hog cholera and the enteritidis groups. The strains of *Bact. pullorum* were derived from epidemics among young fowls and were checked by agglutination tests.

To present the matter briefly, the table shows that all of the "A" strains gave an initial acidity which returned to neutral by the twenty-second day; that the strains of paratyphoid B gave no initial acidity (though the tubes lightened somewhat), and gave an alkaline reaction beginning about the eighth day. By the time the paratyphoids A had returned to neutral, the paratyphoids B showed a moderately strong alkaline reaction (grade 2). The hog cholera and the mouse typhus strain behaved as did the paratyphoids B. The enteritidis strains agreed fairly well with the paratyphoids B, but gave a partial or complete reduction on or before the sixth day. The tests of enteritidis will bear repeating. The pullorum strains showed an initial acidity which passed quickly into a neutral, then to an alkaline

reaction about the thirty-eighth day, sometimes earlier. The strains of *B. avisepticus* did not alter the appearance of the milk in any way during the course of the observations and the control tubes remained essentially unchanged up to the time of evaporation.

The special point to which attention is now called is that notwithstanding the difference which appears between the paratyphoids A and B at the twenty-second day, the difference tends to disappear with the lapse of time. On the thirty-eighth day two of the eight paratyphoid A strains were alkaline; on the fiftieth day three were alkaline; and on the ninetieth day all showed a strong alkaline reaction (grade 3). At this time the only difference between the alpha and beta types was that the beta types showed a slightly stronger alkalinity (grade 4), also by *transmitted* light, a deep wine-red translucency, while the A types showed no translucency. In other words after the lapse of ninety days in litmus milk, the two groups were practically indistinguishable so far as the degree of alkalinity was concerned.

These results appear to suggest that "permanent acidity" cannot be regarded as one of the chief characteristics of *B. paratyphosus A* as stated by Bainbridge (1909); and that initial acidity cannot be regarded as a permanent feature of *B. paratyphosus B* as stated by many writers. It is clear that *both* give the "terminal alkaline" reaction; and the only difference is one of time and degree. In other words there appears to be, in this respect, no fundamental *qualitative* difference between the two bacterial types, but one involving *quantitative* relations only. This circumstance might suggest that, although the litmus milk test may be employed with advantage to differentiate between the two groups, the readings should be taken at a definite time, possibly not earlier than the twelfth day; and for *B. paratyphosus A*, not later than the thirtieth. In other words the test of *B. paratyphosus A* in litmus milk is not the absence of a terminal alkaline reaction, but the presence of a marked initial acidity; while the test for *B. paratyphosus B* is a strong alkaline reaction which usually begins to appear in

milk at a period comparable with the acid-stage of *B. paratyphosus A*. In view of the similarity in reaction after long periods, the translucency ("Aufhellung") test would seem to gain an added significance as a means of diagnosing the A types, although more data are needed on this point.

ADDENDA

After the above note was prepared for press, the paper by Krumwiede, Pratt and Kohn (1917) came to the notice of the writer. The results for the most part correspond, aside from the circumstance that the present writer did not observe the intergradation of the A and B types, with respect to the degree of the alkaline reaction. This may be due to the fact that Krumwiede studied a much greater number of cultures. Krumwiede does not mention the presence or absence of translucency which the present writer is inclined to regard as an important diagnostic feature, separating the A types from the B and enteritidis and hog cholera types quite as distinctly as the initial acid reaction of the A group. It might be of interest to ascertain whether the feature of translucency, if studied in a greater number of cultures such as Krumwiede reports, would show intergradations which the present writer may have missed in the smaller number of strains examined.

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A STUDY OF THE DIPHTHEROID GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO ITS RELATION TO THE STREPTOCOCCI[1]

PART II. CLASSIFICATION OF THE DIPHTHEROID GROUP

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PREVIOUS CLASSIFICATIONS

The difficulties of biologic classification among organisms showing such slight differentiation as the bacteria, have long been noted by bacteriologists. Attempts to arrange assemblages of such organic forms under the captions of species have not met with uniform success, partly on account of overlapping characteristics, and partly on account of the various transitions which this subjective term "species" has experienced from time to time. It is much more practicable to take refuge behind the word "group" as Dr. Prudden (1898) long ago remarked.

Biologically it is doubtful if toxin production on the part of an organism should be considered as a major characteristic, nevertheless from the standpoint of pathology and medicine, it is a quality of first importance, and thus considered indicates a position "sui generis" for the Klebs-Loeffler bacillus. I merely cite this as a concrete difficulty. An adequate classification must conform to the biologic conception, yet it should defer as far as possible to the more utilitarian claims of practical medicine and pathology.

There have apparently been very few attempts to develop a comprehensive, systematic arrangement of the diverse members of the diphtheroid group. The term pseudo-diphtheria

[1] A thesis for the degree of Doctor of Public Health.

bacillus has been inadvertently applied to all atypical diphtheria organisms. This is generally recognized as unfortunate, as the term was already preempted for the organism described by Loeffler (1887) and Hoffman-Wellenhoff (1888) and now commonly known as *B. Hoffmanii*.

For some years after the discovery of this bacillus there followed a controversy regarding its relation to the Klebs–Loeffler organism. The monists held that the Hoffman bacillus was an attenuated variety of the true diphtheria bacillus, while the dualists regarded them as distinct and separate species. It cannot be said that the question is fairly settled yet, although the present trend of opinion is decidedly opposed to the view that the organisms are separate species in the sense of being totally unrelated. In view of the immense amount of work that has been done, the contention of Flexner and others that *B. Hoffmanii* or the pseudo-diphtheria bacillus is a true mutant seems logical.

It was soon apparent that the pseudo-diphtheria bacillus was by no means a single race of organisms, and that in all probability there existed a large number of strains of diphtheria-like bacilli, which are identical with neither the Klebs-Loeffler nor the *B. Hoffmanii*. Although there have been desultory contributions to the literature of this subject for the past twenty years, bacteriologists in general have lapsed into a state of apathy regarding the pathogenic properties of diphtheria-like microörganisms. True, this negligent attitude has been unsuccessfully assailed from time to time, by workers whose experience with the group had convinced them that all its members did not merit a wholesale relegation to the saprophytic scrap-heap. The investigations of Alice Hamilton, Ruediger, Hektoen, Rosenow and others have demonstrated beyond peradventure, that certain strains of this diverse group are capable under certain conditions of showing definitely pathogenic properties.

The lethargic attitude regarding the diphtheroids suffered a shock as the result of a series of investigations begun by Frankel and Much in 1910 and continued by de Negri and Miermet, Bunting and Yates, Rosenow and many others. The first

mentioned authors studied exhaustively thirteen cases of Hodgkin's disease, and were able to recover from the sediment of the lymph glands previously treated with antiformin, certain pleomorphic non-acid-fast bacilli which they did not succeed in cultivating. Frankel and Much, as a result of their studies came to the conclusion that this organism was either an attenuated or a special variety of the tubercle bacillus.

Shortly after this paper appeared, de Negri and Miermet and later Bunting and Yates succeeded in cultivating this organism which had the characteristics of a diphtheroid, and was named by the latter authors *B. corynebacterium Hodgkini*. They claimed to have reproduced with it the characteristic lesions of Hodgkin's disease in monkeys although as yet the contention lacks confirmation. As a result of these investigations a new interest has been awakened in this group, which has manifested itself in the character and number of contributions to the recent literature.

These contributions have been directed mainly toward one of the more pressing exigencies of the situation, namely, the discovery of the *B. Hodgkini* in normal and non-Hodgkin's lymph glands as well as in other locations. As a result a large number of diphtheroids of various types have been found, but owing to the lack of any classification of the group considered as adequate, the various observers state with one accord that they are unable to harmonize their various findings with those of each other. Arbitrary groupings and provisional classifications, based largely on expediency or on some minor characteristic have failed to bring order out of the confusion which at present reigns.

B. xerosis also labors under a variety of interpretations. Some make this species identical with the avirulent diphtheria bacillus, while the term is restricted by others to the Kuschbert-Neisser (1884) organism originally thought to be the cause of xerosis, but later found to be a more or less common inhabitant of the normal conjunctival sac.

All through the literature one finds instances of diphtheria-like bacilli receiving specific designations based on a variety

of minor characteristics, such as morphology, or action on gelatin, or perhaps on potato, or pigment production, appearance on serum, or some inadequate combination of these characters, many of which can easily be modified. Another source of confusion has been the results of the sugar reactions in the hands of various observers. For a long time strains were tested on glucose only and naturally it is difficult to correlate such descriptions with those which have been based on six or eight different sugars. Little attention has been paid to immunologic characteristics as a criterion for classification.

The best classification of the diphtheroids that I have encountered is undoubtedly the one made by Morse (1912 a). She divides the entire diphtheria family into two main groups: the Klebs-Loeffler bacillus and the diphtheria-like bacilli or diphtheroids. Of these latter she recognizes four sub-groups which are the following:

Group A. This is the largest numerically. The bacilli correspond to the "organism x" described by Hoag from the Danvers State Hospital. It is a medium sized bacillus, showing solid, barred and wedge forms, with abundant but small and imperfect granules. On serum it produces a heavy, confluent, glistening growth with a characteristic salmon-pink color. It ferments glucose and sucrose, but not maltose or glycerin.

Group B. The organisms of this group are usually larger than those of Group A, and thick forms with clear cut bars predominate. Neisser's granules are very large and irregular. The growth on serum is heavy and varies in color from white to yellow. It is often noticeably dry and granular. Glucose is always fermented, maltose and glycerin usually, but not sucrose.

Group C. This, the smallest group, is differentiated primarily by its slow, scanty, colorless or white growth. Morphologically, the organisms resemble those of Group B. They always acidify glucose, and both sucrose and maltose usually.

Group D. Composed of thick, small, straight bacilli, often barred and wedge-shaped, showing no granules. The growth on serum may be scanty or abundant and is white or yellowish white in color. They do not act upon glucose, maltose, glycerin, or sucrose.

Morse names these four groups as follows: "Group A, *B. Hoagii:* Group B, *B. flavidus:* Group C, *B. xerosis* and Group D, *B. Hoffmanii.*" I have retained this terminology in my own classification, and have confirmed the validity of these various groups as they stand.

DISTRIBUTION OF THE DIPHTHEROID GROUP

The diphtheroids are among the most ubiquitous of all bacterial organisms. They are to be found not only in the bodies of animals and man, but also have a wide distribution in nature. R. O. Neuman (1902) and Sudeck (1896) first found them in the air. Wade and Harris (1915) and Torrey (1916) have made definite experiments on this point with positive results. Wade exposed 18 blood-agar plates to the air and recovered 45 colonies of diphtheroids. He has also isolated these organisms from the urine and feces. I have myself isolated them from the air of various laboratories. McNaught (Graham-Smith, 1908) has obtained them from water. McCambell (Graham-Smith, 1908) and Bergey (1904) have found them in milk.

It is of great interest to know that diphtheroids have been found in practically all the organs of the human body in either pure or mixed culture, and in both health and disease. Since the claim of their etiological relation to Hodgkin's disease, there have been several reports regarding their presence both in normal lymph glands and in gland conditions bearing no relation to Hodgkin's disease. Harris and Wade (1915) have written a report on the "Wide distribution of diphtheroids and their occurrence in various lesions of the human tissues." Besides normal and pathological lymph glands (not Hodgkin's) they have isolated diphtheroids from leiomyomata, fibroma and hepatic cancer, as well as from tuberculosis of lymph glands and lymphosarcoma. Fox (1915 b) has isolated them from the normal and diseased lymph nodes in many cases. Several strains came from lymph glands draining enlarged joints. Torrey (1916) in a special article on lymph gland bacteria, cultured 30 strictly lymph gland conditions and 10 conditions in which

the lymph glands were secondarily involved. He recovered the organisms from one type or other in 22 of the cases. Bloomfield (1915) reports a similar experience. Hoag (1907) finds the organisms in almost every part of the body but mainly in the respiratory tract. Bergey (1904) has found them in spontaneous abscesses in animals, and in cutaneous suppurations and in tumors. Hoag (1907), and Orr, Rows, and Robertson (1910) found these organisms in the central nervous system. J. A. Langford (1914) and Bunting and Yates (1914) found diphtheroids in the spleen in Banti's disease. Hektoen (1901) found them in large numbers in the liver, Axenfeld (1899) and others in the conjunctival sac, Walsh (1899) in the skin, Hall and Stone (1916) in the lymph glands of horses, sheep and calves. They have been found by many observers in the urethra (Hine, 1913) in the vagina (by Voigt, personal communication) and in the bladder (Townsend 1905). Rosenow (1915) has isolated them from the blood, joints, glands, lungs, skin, parotid gland, ganglia of the central nervous system and many other locations. I have myself isolated them from many locations in the body, but especially from the upper respiratory tract, skin, urethra, and lymph glands, less often from joints, blood, lungs and spinal fluid. This list could be indefinitely extended, but as no purpose could be served by so doing, I shall briefly summarize this topic by saying that the most common situations in which diphtheroids may be found in the human body are the mucous surfaces and skin, but a perusal of the references cited shows that no portion of the organism is exempt from them.

MORPHOLOGY

The size and shape of diphtheria-like organisms is subject to an almost infinite number of variations. Some of the subgroups have a fairly constant shape while in others the pleomorphism is so marked that almost any conceivable shape may be assumed as the environmental factors are altered. The detailed classification of diphtheria bacilli by Wesbrook includes all the ordinary and many of the rarer forms encountered in

the throat. The shapes most frequently met with are embraced under his three divisions; first the granular, second the barred and third the solid type. Type I shows spherical or oval metachromatic granules, usually located at the ends of the bacilli although they may be noted elsewhere. The protoplasm stains faintly (Loeffler's). Type II is the barred or segmented type and is characterized by great regularity of staining. The bars may be so arranged as closely to resemble streptococci and indeed may be mistaken for them. Cobbett (1901) designates this form as a separate morphological type. Neisser (1897) mentions a streptobacillus which is not unlike the Klebs-Loeffler organism. This streptococcoid form is not one that has received much recognition in the literature, but in the light of the relation between the streptococcus and the diphtheroid groups to be discussed later on, I believe that such forms merit a more careful consideration. The number of the segments or bars may be from 3 to 20 and they may or may not be metachromatic. The third or solid type is non-granular, may have almost any size and shape, and often appears as a diplobacillus. The different types ordinarily vary in thickness from 0.25 to 2 microns and in length from 1 to 8 microns.

The varied morphology of the diphtheroid group and the conditions which modify it, have been the subject of much discussion in the past. The question has hinged largely on the stability of the morphological and toxic types under varying conditions. A. Williams (1902) makes a thorough revision of this whole subject and in addition repeats the previous experiments as well as contributes new ones with a view to clearing up the question as to the fixity of biological types. Her conclusions are as follows:

> Though some cultures change on some media, each changes in its own way and each culture still has its own individuality. Even though the morphology of a culture may be radically changed by alteration of media, etc., nevertheless when transplanted to the same media and under the same conditions that conduced to the establishment of the type, the original morphology will return.

Morse (1912) also believes that the morphological individuality of a culture is retained tenaciously under good conditions. She studied 295 strains and was able to correlate definitely several morphological types with other fixed characters such as sugar fermentation, etc. Both authors say however that the strain must be observed through several generations and a constant morphology obtained, before attempting cultural changes to induce marked pleomorphism.

My own observations are for the most part in accord with these workers although there is one sub-group which has a pleomorphism so extreme that it will not conform to the above restrictions. Among the strains that I have studied there is a certain percentage described as *B. enzymicus* in Part I (Journal of Bacteriology, March 1917) which undergo a very remarkable change of form which remains quite constant and which is not brought back to type merely by restoring the original conditions. A barred, fairly long bacillus of this kind can be changed to a typical diplococcus or to a long-chained streptococcus (diplostreptococcus, Part I, pp. 84–85, Journal of Bacteriology, March, 1917). When this form has developed it remains quite constant and although it is possible to cause it to assume, a bacillary form again, this is accomplished only with great difficulty. These forms are not involution forms in the ordinary sense of that term and are to be clearly distinguished from the ordinary coccoid form of diphtheroids and other bacilli. The coccoid forms are usually single, vary wonderfully in both size and staining characters and are almost always metachromatic. They take the stain very intensely and irregularly, and generally appear under rather adverse conditions. One usually has no difficulty in distinguishing them from true cocci. The streptococcus form is not distinguished from the true cocci by ordinary staining methods. Some of the strains which I have studied showing these characteristics are numbers 1, 3, 11, 13, 14, 16, 26, and 33 (see table 2).

INVOLUTION

This is a very common phenomenon among the different members of the diphtheroid group, being most frequent and

8	Ear	+++	− ++	−	+	ight yellow	Opaque moist confluent growth
19	Ear	+++	± +	−	−	−	As on Loeffler's
32	Skin; erythema multiforme macule	+++	− ±	−	−	+	Very heavy, moist, confluent growth
34	Ear	Luxuriant growth	− ±	Rods stain; no growth	−	−	Very heavy, moist confluent growth
43 (x 320)	Spleen; Banti's disease	±	− ±	Rods stain; no growth	−	−	Moderate grayish white and confluent colonies
21	Ascitic fluid	+++	− ++	−	+++	±	Heavy, moist growth. Takes place only between 20° and 30° C.
10	Throat	Scant	± ++	−	−	−	Negative
24	Throat post-diphtheritic	±	− ++	−	−	−	Moderate semi-transparent
25	Throat post-diphtheritic	±	− ++	−	−	−	Moderate, semi-transparent
26	Guinea-pig, lung abscess	±	− ++	−	++	±	Moderate, semi-transparent
31	Nasal septum; non-syphilitic perforation	Scanty growth	− ++	−	−	−	Very scant white semi-transparent smear
44 (x 321)	Spleen, chronic leukemia	±	− +	−	−	−	Bacillus; thin, moist film
12	Eye;conjunctiva	Slight	± +	+	±	±	Very scant dry growth, not increasing at 25°C.

* One per cent HCl in 20 per cent ethyl alcohol and two per cent HCl in 50 per cent ethyl alcohol
† All cultures sent me by Dr. Bunting of Madison, Wisconsin, are designated by letter x.

Colonies, dark center and light border	As on glucose	Heavy sediment; supernatant clear	Acid. No coagulation	Heavy moist yellow growth	Mostly in upper part of tube	+
As on Loeffler's	Luxuriant opaque moist growth	Diffuse heavy growth with sediment	–	Heavy moist yellow growth	As agar. No liquefaction	–
As on agar; yellow pigment	Heavy, moist growth; liquefies in 48 hours. Complete in 6 days	Heavy turbid growth	Slight reddening of litmus	++	White growth in upper part of tube	–
As agar	As agar	Heavy precipitate. Supernatant clear	No acid	Luxuriant growth	–	+
Semi-transparent moist confluent	As blood	Luxuriant diffuse with pellicle after 3 days	–		–	–

Sub-Group B. liquefaciens

Yellow pigment	Scant growth liquefies in 4 days at 30°C.	Finely flocculent after 4 days, becomes diffuse and luxuriant	Acid	Moist; elevated yellowish-white growth	Nailhead culture, progressive liquefaction	+-

Sub-Group B. Hoffmanii

Slow growth; transparent discrete colony	Moderate moist growth in 5 days	–	–	–	Delicate growth in upper half of tube	+
Moderate opaque confluent	As on blood	Heavy sediment; supernatant clear	–	–	Scant growth extending to bottom of tube	–
No increase at room temperature	Fine discrete dew drop colonies	Heavy sediment; supernatant clear	–	–	Scant growth extending to bottom of tube	Sligh +
As on agar	Fine discrete dew drop colonies	Heavy sediment; after 4 days becomes heavy	Slight reddening; no coagulation	–	Scant growth extending to bottom of tube	–
Transparent discrete colonies in 24 hours; opaque and elevated in 48 hours	Discrete colonies; moist growth	Scant sediment; supernatant clear	–	–	Very scant; greater in bottom of tube	+
As agar	As agar; slow growth	Bacillus; fine granular sediment.	–		Small white discrete colonies	–

Sub-Group B. Ruedigeri

Sub-Group B. xerosis

As glucose agar	Fairly luxuriant moist growth after 3 days	Very few fine floccules at the bottom of the tube	Decided acid; coagulation on boiling	–	–	Sligh +

l were used to decolorize the carbol-fuchsin. See page 280.

n up- rt of	+	++	−	This strain apparently represented another type of diphtheroid but in reality proved to be a variant of strain 34 that grew first at room temperature which strain 8 refused to do.
No action	−	++	−	
rowth er part e	−	++	−	
-	+	−	−	
-	−	++	Splenic fibrosis in dogs (Bunting)	Dr. Bunting claims to have produced Banti's disease in dogs with this strain. It is noteworthy that its sugar reactions are not at all similar to his Hodgkin's strains.
l cul- progres- quefac-	++	+++	−	This strain would not grow at 37°C. Optimum temperature 30°C.
growth r half	+	+++	−	
owth ing to n of	−	+	−	
owth ing to n of	Slight +	++	−	
owth ing to n of	−	++	−	This strain grew at 37° C. after isolation, but later would grow only at 30°C.; also grew in chains of streptococci.
nt; r in n of	+	+	Rabbits lose 30% weight	This strain was isolated in pure culture from a chronic nasal ulcer which was non-syphilitic.
hite e colo-	−	++	−	
-	Slight +	+	−	It is noteworthy that this strain could not be made to develop coccoid forms or grow diffusely in broth.

TABLE 2

Characteristics of bacilli of the diphtheroi

Sub-group B. enzymicus

STRAIN NO.	SOURCE	VIGOR GROWTH	*ACID FAST / GRAM STAIN	ANTIFORMIN RESISTANT	INVOLUTION FORMS	PIGMENT	GLUCOSE AGAR	BLOOD AGAR	LOEFFLER'S SERUM	GLUCOSE BROTH
1	Human lung	±	± / ±	+	+++	−	Bacillary form shows pin-point colonies; Coccus form. heavy white moist growth	As on glucose	Fine, moist, confluent growth	Bacillary form, granular sediment; coccus form, diffuse luxuriant
2	Human blood and urine	±	+ / ++	−	++	Reddish-brown blood agar	Anaerobic	Reddish-brown on blood agar; old culture	Same as blood-agar	Scant sediment
3	Lymph gland Hodgkin's disease	±	+ / ±	+	+++	Yellowish-brown on serum	Discrete moist, slowly growing white colony	Moist confluent, yellowish-red in four days	As on blood	Moderate sediment
9	Unknown	++	± / ++	−	+	−	Very scant opaque film	Scant transparent growth	As on blood	Scanty sediment
11	Normal throat	Slight	+ / ±	Rods stain; no growth on culture	+++	−	−	Very scant discrete dew-drop colony	As blood agar	Scant sediment in bottom of tube
13	Urethra, acute gonorrhoea	Slight	± / ±	Rods stain; culture negative	++	−	−	Spreading; transparent colony opaque in center	Only grew after repeated transplants on blood agar	Very few fine floccules at the bottom of the tube
14	Acute gonorrhoea	Slight	± / ±	Rods stain	++	+	Almost invisible dewdrop growth	Spreading; transparent colony, opaque in center	Only grew after repeated transplants on blood agar	Very scant sediment
16	Nose acute rhinitis	Slight ±	++ / ++	−	+++	−	Scant growth; colonies have dark center and light border	Dewdrop colonies	Only grew after several transplants on rabbit serum	Very scant sediment
33	Urethra gonorrhoea	Scanty ±	− / ±	Rods stain; no growth	−−	−	−	Very fine pin-point colonies	As blood agar	−
37 (x† 325)	Lymph gland Hodgkin's disease	±	− / ++	Rods stain; no growth	−−	−	Bacillus negative	Scant moist film yellow pigment	As blood agar. colonies discrete	Slight, as bacillus; nebulous deposit
38 (x 324)	Lymph gland Hodgkin's disease	±	− / ++	Rods stain; no growth	−−	−	Bacillus negative	Scant moist film, yellowish-red pigment	As blood agar; colonies discrete	Slight, as bacillus; nebulous deposit
39 (x 323)	Lymph gland Hodgkin's disease	±	− / ±	Rods stain; no growth	−−	−	Bacillus negative	Scant moist film, yellowish-red pigment	As blood agar; colonies discrete	Slight, as bacillus; nebulous deposit
40 (x 322)	Lymph gland Hodgkin's disease	±	+ / ±	Rods stain; no growth	−−	−	Bacillus negative	Scant moist film, yellowish-red pigment	As blood agar; colonies discrete	Slight, as bacillus; nebulous deposit
41	Urethra; gonorrhoeal rheumatism	++	− / +	Rods stain; culture negative	−−	−	Firm isolated pin-point colonies	Transparent discrete colonies; slow	As agar	Fine granular deposit in bottom of tube, in 24 hours
42	Acne; comedo; skin	+	− / ±	−	−−	−	Moist confluent; appears first at bottom tubes	As agar	As agar	Luxuriant diffuse in 72 hours in coccus form
45 (x 328)	Pseudo leukemia Lymph glands	±	− / +	Rods stain	−−	−	Bacillus; thin, moist film	As agar	As agar, slow growth	Bacillus; fine, granular sediment
35 (T 337)	Uterus, guinea pig; infective abortion	++	− / ++	−	−	−	Medium white confluent dry growth	Spreading adherent dry wrinkled colonies	As agar	Heavy precipitate; pellicle in 48 hours, very friable
										Sub-Group B. Hoagii
4	Hodgkin's lymph gland	+	− / +	−	±	−	Rather moist, semi-transparent growth; transparent on standing	As on glucose agar	As on glucose agar	Scant sediment turbid in 72 hours
5	Hodgkin's lymph gland	+	+ / +	−	−	−	Rather moist, semi-transparent growth; transparent on standing	As on glucose agar	As on glucose agar	Scant sediment turbid in 72 hours
6	Spinal fluid	++	± / ++	−	−	−	Abundant, moist, thick growth in 18 hours	Semi-transparent colonies or opaque colonies in 48 hours	Discrete opaque colonies 48 hours	Heavy sediment; supernatant clear
7	Lung	++	± / +	−	−	−	Abundant, moist, thick, growth in 18 hours	As on glucose	Discrete opaque colonies 48 hours	Heavy sediment; supernatant clear

f the diphtheroid group
nzymicus

GLUCOSE BROTH	LITMUS MILK	POTATO	GELATIN STAB	INDOL	NITRITES	VIRULENCE	REMARKS
Bacillary form, granular sediment; coccus form, diffuse luxuriant	Acid, coagulation	Negative	Moderate growth; no liquefaction	SL +	+	++ Rabbits, mice, guinea-pigs	Special features were discussed in Part I.
Scant sediment	No acid	–	No surface growth, pos. in lower part of tube	–	+	– – Rabbits, guinea pigs	Fuller data under Part III.
Moderate sediment	Slight acid; no coagulation	Luxuriant white growth	Luxuriant reddish-brown growth; viability prolonged	+	+	Rabbits die of inanition	Rabbits injected intraperitoneally and subcutaneously; developed progressive cachexia and died. No lesions. Strain soon lost its pathogenicity on artificial media.
Scanty sediment	Slight acid; no coagulation	Scant dry yellowish film	Fairly luxuriant to bottom of tube	–	–	–	
Scant sediment in bottom of tube	Slight acid; no coagulation	–	–	–	++	–	This strain was isolated on glucose ascitic agar and morphologically was *B. Hoffmanii*. It proved to be *B. enzymicus*
Very few fine floccules at the bottom of the tube	–	–	–	+	+	For rabbits, sepsis, thrombosis	This strain is another example of pathogenicity in the *B. enzymicus* group. Young rabbits were especially susceptible.
Very scant sediment	Red; no coagulation	Very few fine opaque colonies	Single colonies along upper half of inoculation; they have a dark center	–	+	+ For young rabbits	Produces thrombosis in small veins of skeletal muscles by localizing in the endothelium of vessel wall. Hemorrhage and perivascular infiltration. Cholecyctitis, sepsis. Virulence is soon lost.
Very scant sediment	–	–	Scant growth in upper half of tube	–	++	– For rabbits and guinea pigs	
–	Alternates between red and blue	–	–	–	–	–	
Slight, as bacillus; nebulous deposit	Acid coagulation on boiling	–	Very slight growth; no liquefaction	–	+	–	These cultures are the strains of *B. Hodgkini* (Dr. Bunting). When I received them they grew with difficulty. By method described in Part I they developed luxuriant growth in the coccus form very similar to strain 1.
Slight, as bacillus; nebulous deposit	Acid coagulation on boiling	–	Very slight growth; no liquefaction	–	+	–	
Slight, as bacillus; nebulous deposit	Acid coagulation on boiling	–	Very slight growth; no liquefaction	–	+	–	
Slight, as bacillus; nebulous deposit	Acid coagulation on boiling	+	Very slight growth; no liquefaction	–	+	–	
Fine granular deposit in bottom of tube, in 24 hours	Acid and coagulation	–	Very slight	–	+	–	This strain was isolated in pure culture from the urethra of a case of gonorrhoeal rheumatism. The case received marked benefit from vaccines made from it.
Luxuriant diffuse in 72 hours in coccus form	Acid and coagulation	–	More marked in bottom of tube; fairly heavy	+	++	Marked loss in weight in rabbits	It is not usual to find such a widely fermenting organism in the skin. Case was cured by vaccines made with this organism.
Bacillus; fine, granular sediment	Acid, coagulated on heating		Slight white discrete colonies	+	+	–	
Heavy precipitate; pellicle in 48 hours, very friable	–	–	–	–	+	Produced rapid emaciation and death in rabbit, no lesions	This organism was proven to have caused an epizootic of abortion in guinea-pigs.

B. Hoagii

Scant sediment; turbid in 72 hours	Slight acid; media clear Bordeaux-red color	Yellow luxuriant growth	Delicate opaque growth; liquefaction very slow	–	+++	–	These strains gave a moderate white growth on agar, but on standing in the ice-chest the growth became perfectly transparent
Scant sediment; turbid in 72 hours	Slight acid; media clear Bordeaux-red color	Lemon yellow moist colonies	Delicate opaque growth; liquefacation very slow	–	++	–	As above. It is not usual for *B. Hoagii* to liquefy gelatin.
Heavy sediment; supernatant clear	Alternated between red and blue; no coaglation	Discrete colonies of dirty yellow color	Slight growth in upper one-third of tube	–	+	–	
Heavy sediment; supernatant clear	Alternated between red and blue; no coagulation	Dirty dry grey growth	Extends two-thirds way to bottom of tube	–+	++++	–	

8	Ear	+++	− ++	−	+	ight yellow	Opaque moist confluent growth	Colonies, dark center and light border	As on glucose	Heavy sediment; supernatant clear
19	Ear	+++	± +	−	−	−	As on Loeffler's	As on Loeffler's	Luxuriant opaque moist growth	Diffuse heavy growth with sediment
32	Skin; erythema multiforme macule	+++	− ±	−	−	+	Very heavy, moist, confluent growth	As on agar; yellow pigment	Heavy, moist growth; liquefies in 48 hours. Complete in 6 days	Heavy turbid growth
34	Ear	Luxuriant growth	− ±	Rods stain; no growth	−	−	Very heavy, moist confluent growth	As agar	As agar	Heavy precipitate. Supernatant clear
43 (x 320)	Spleen; Banti's disease	±	− ±	Rods stain; no growth	−	−	Moderate grayish white and confluent colonies	Semi-transparent moist confluent	As blood	Luxuriant diffuse with pellicle after 3 days
										Sub-Group B. liquefaciens
21	Ascitic fluid	+++	− ++	−	+++	±	Heavy, moist growth. Takes place only between 20° and 30° C.	Yellow pigment	Scant growth liquefies in 4 days at 30°C.	Finely flocculen after 4 days, becomes diffuse and luxuriant
										Sub-Group B. Hoffmanii
10	Throat	Scant	± ++	−	−	−	Negative	Slow growth; transparent discrete colony	Moderate moist growth in 5 days	−
24	Throat post-diphtheritic	±	− ++	−	−	−	Moderate semi-transparent	Moderate opaque confluent	As on blood	Heavy sediment; supernatant clear
25	Throat post-diphtheritic	±	− ++	−	−	−	Moderate, semi-transparent	No increase at room temperature	Fine discrete dew drop colonies	Heavy sediment; supernatant clear
26	Guinea-pig, lung abscess	±	− ++	−	++	±	Moderate, semi-transparent	As on agar	Fine discrete dew drop colonies	Heavy sediment; after 4 days becomes heavy
31	Nasal septum; non-syphilitic perforation	Scanty growth	− ++	−	−	−	Very scant white semi-transparent smear	Transparent discrete colonies in 24 hours; opaque and elevated in 48 hours	Discrete colonies; moist growth	Scant sediment; supernatant clear
44 (x 321)	Spleen, chronic leukemia	±	− +	−	−	−	Bacillus; thin, moist film	As agar	As agar; slow growth	Bacillus; fine granular sediment.
										Sub-Group B. Ruedigeri
										Sub-Group B. xerosis
12	Eye;conjunctiva	Slight	± +	+	±	±	Very scant dry growth, not increasing at 25°C.	As glucose agar	Fairly luxuriant moist growth after 3 days	Very few fine floccules at the bottom of the tube

* One per cent HCl in 20 per cent ethyl alcohol and two per cent HCl in 50 per cent ethyl alcohol were used to decolorize the carbol-fuchsin. See p

† All cultures sent me by Dr. Bunting of Madison, Wisconsin, are designated by letter x.

eavy sediment; supernatant clear	Acid. No coagulation	Heavy moist yellow growth	Mostly in upper part of tube	+	++	–	This strain apparently represented another type of diphtheroid but in reality proved to be a variant of strain 34 that grew first at room temperature which strain 8 refused to do.
iffuse heavy growth with sediment	–	Heavy moist yellow growth	As agar. No liquefaction	–	++	–	
eavy turbid growth	Slight reddening of litmus	++	White growth in upper part of tube	–	++	–	
eavy precipitate. Supernatant clear	No acid	Luxuriant growth	–	+	–	–	
uxuriant diffuse with pellicle after 3 days	–	–	–	–	++	Splenic fibrosis in dogs (Bunting)	Dr. Bunting claims to have produced Banti's disease in dogs with this strain. It is noteworthy that its sugar reactions are not at all similar to his Hodgkin's strains.
quefaciens							
nely flocculent after 4 days, becomes diffuse and luxuriant	Acid	Moist; elevated yellowish-white growth	Nailhead culture, progressive liquefaction	++	+++	–	This strain would not grow at 37°C. Optimum temperature 30°C.
. Hoffmanii							
–	–	–	Delicate growth in upper half of tube	+	+++	–	
eavy sediment; supernatant clear	–	–	Scant growth extending to bottom of tube	–	+	–	
eavy sediment; supernatant clear	–	–	Scant growth extending to bottom of tube	Slight +	++	–	
eavy sediment; after 4 days becomes heavy	Slight reddening; no coagulation	–	Scant growth extending to bottom of tube	–	++	–	This strain grew at 37° C. after isolation, but later would grow only at 30°C.; also grew in chains of streptococci.
ant sediment; supernatant clear	–	–	Very scant; greater in bottom of tube	+	+	Rabbits lose 30% weight	This strain was isolated in pure culture from a chronic nasal ulcer which was non-syphilitic.
acillus; fine granular sediment.	–		Small white discrete colonies	–	++	–	
. Ruedigeri							
. xerosis							
ry few fine floccules at the bottom of the tube	Decided acid; coagulation on boiling	–	–	Slight +	+	–	It is noteworthy that this strain could not be made to develop coccoid forms or grow diffusely in broth.

uchsin. See page 280.

most varied among the granular and barred types, while it is more unusual among the solid types, although some of them show it. Bunting (1913), de Negri and Miermet and many others emphasize the extreme pleomorphism of the so-called *B. Hodgkini*. Club forms and segmented forms are common, while almost all observers agree that the coccoid forms are so confusing that it is only by repeated platings that one may be sure that a pure culture is obtained. Fox (1915) shows photomicrographs of diphtheroids from the lymphatic glands, mostly of Hodgkin's cases. Some of these develop long clubbed metachromatic forms, others assume very long sinuous filamentous forms. Others occur as threads, some resemble short chains (streptococcoid forms) while some are fused and branched.

Neisser (1884) and Eyre (1896) speak of the marked clubbing of the xerosis bacillus on dried glycerin serum. Rosenow (1915) mentions the remarkable involution forms of the causative diphtheroid of erythema nodosum. In ascitic-glucose broth only bacillary forms appeared, while on blood-agar plates, he recovered small diplococci in short chains.

Strain 1 in the series under investigation in this study showed very similar characteristics. In ascitic glucose broth, growth was slight with no clouding of the media. No coccoid forms developed under these conditions. The bacilli were barred and granular. The granules stained intensely with Loeffler's stain while the protoplasm stained faintly. Branched forms were numerous after four days, and on several occasions the most extraordinary filamentous network was seen; this branching form of the organism resembled closely certain of the streptothrices. On the nodal points of the network one or more granules of heavily staining chromatin were to be seen. These granules were of varying size and often stained metachromatically. The network itself stained faintly, and resembled debris but on closer examination and differentiation proved to be a direct outgrowth of the organism. The shorter fused forms described by Park and Williams (1910) were also not uncommon in this medium and were usually associated with the more complex structure just described.

On blood-agar slants the ordinary coccocid forms developed. The size of the cells varied between wide limits. They grew singly or in clumps, seldom as diplococci and were spherical or ovoid in shape. They stained intensely in Gram or Loeffler's stains and metachromatism of varying grades was the rule. With careful differentiation or double staining, marked chromatic irregularities appear. A more complete description of this strain will be found in Part I, Journal of Bacteriology, March, 1917.

GROUPING

Most characteristic among the various possibilities of arrangement is the palisade grouping. This was described by the early writers Neisser (1897), Prochaska (1897), and others, and has been emphasized by the more recent contributions to this subject, Park and Williams (1910), Teoumin (1913). V-shapes and diplobacilli are also very common. It is also usual to see diphtheroids in clumps or in tangled masses.

STAINING

As a general thing diphtheria like-organisms stain readily and intensely with the simple anilin dyes. Occasionally one encounters a strain of granular bacilli which reacts faintly to Loeffler's but in such instances Gram's stain produces excellent results.

With Gram's stain one finds all gradations of reaction. It is usually stated that all diphtheroids are Gram-positive. Prochaska (1897) says that "decolorization must not be too energetic." Hamilton (1907) describes a Gram-negative diphtheroid. De Witt (1912) speaks of Gram-variable diphtheroid bacilli. I have referred to the effect of old or dry media, the age of the culture, etc., on the intensity of the Gram stain with the so-called *B. Hodgkini*, (Mellon, 1915). "In some cultures all grades of reaction to this stain might be seen; in others, the entire culture was finally decolorized." "Coccoid forms usually take the stain more intensely than the bacilli." This has also been observed by Steele (1914) and others.

No matter how pleomorphic a strain may be, the chromatin granules are practically always Gram-positive, while the body of the bacillus or projections therefrom are usually Gram-negative, if the culture is relatively young. The filamentous forms which I have described above as well as in another communication, are always Gram-negative (Mellon, 1915). These variations are much more noticeable in the granular and barred types than in the solid ones. So although the diphtheroids can properly be classed among the Gram-positive bacilli, the limiting conditions must be very strictly adhered to, and even then some strains will be doubtful.

The principle holds equally well regarding the property of "fastness" to acids. Diphtheroids are usually described as being non-acid-fast, and as a general proposition this is true. Nevertheless Wade and Harris (1915) believe that some strains are acid-fast. They class *B. smegma* among the diphtheroids and its reaction in this regard is well known. In addition they have isolated a diphtheria-like organism from the mesoappendix which liquefied blood serum and gelatin, was Gram-positive and acid-fast. Wolbach and Honeij (1914) report "partial acid fastness of a diphtheroid isolated from leprosy lesions." The granular portions of these bacilli are fast to 2 per cent H_2SO_4 followed by 95 per cent alcohol; and 3 per cent HCl in 95 per cent alcohol, reagents acting for 30 seconds. Similar treatment completely decolorized the Klebs-Loeffler and mixed cultures of cocci and bacilli from the throat.

Kedrowski (1901), Bayon and Williams (Harris and Wade, 1915) have cultivated diphtheroids from leprosy, each regarding his culture as a stage in the life cycle of the Hansen bacillus. Kedrowski assumes a mutation from a non-acid-fast to an acid fast organism because he recovered the latter after injection of the former. Campana and Babes (1910) thought Kedrowski was working with mixed cultures since they could not confirm his results. Duval and Duval and Harris also quoted by Harris and Wade have not been able to repeat these experiments. Dr. Eggers tells me that Kedrowski's work has been recently confirmed. Out of 45 colonies of diphtheroids on blood-agar plates

exposed to the air, Harris and Wade (1915) recovered 7 which were distinctly acid-fast.

I have experimented with quite a number of strains in this regard. I used as a routine 1 per cent HCl in 20 per cent ethyl alcohol and 2 per cent HCl in 50 per cent ethyl alcohol as a decolorizing agent. My results are tabulated in table 2. Out of 37 strains treated with 1 per cent HCl in 20 per cent alcohol for one and one-half minutes following previous treatment with carbol-fuchsin, I found that 13 strains retained the stain very definitely. The remaining strains were either decolorized or doubtful, and seven were completely decolorized. Six strains resisted 2 per cent HCl in 50 per cent alcohol. The granules held the stain tenaciously but often the body of the organism was pink also. The results were fairly constant on Loeffler's medium and 2 per cent glucose-agar but all strains that would grow on potato were either negative or doubtful. More constant results are claimed by Rehr (1915) using alcohol-acetone to decolorize the bacilli previously stained by Gram. He claims to be able to diagnose a much larger percentage of cases of true diphtheria by this means.

Here again is seen a lack of constancy in reaction, depending not only on the individuality of the strain but also on the chemical constitution of the medium. My experience with the staining properties of this group gives me the impression that the diphtheroid protoplasm is very labile, very dependent on factors of which we know little, and which we cannot control. For this reason I am rather skeptical about drawing very definite conclusions of any kind from evidence which has as its basis the retention of a stain by such an ephemeral matrix.

The polychromasia or metachromasia of various members of the diphtheria group has long been known and commented upon. It is quite characteristic for members of the granular group to exhibit reddish or magenta colored chromatin. Under certain conditions these granules may become a very brilliant red even when a pure Loeffler's stain is used. A. Williams (Park and Williams 1910) believes "that the metachromatic masses occurring in involution forms of *B. diphtheriae* represent

a primitive sexual process, a sort of autogamy."[2] She believes that disturbance of the culture at intervals facilitates this process. I have been able to verify this observation although it occurred as a result of routine examination of a culture in broth. Strain number 16 of my series grew faintly in ascitic broth and only in the bottom of the tube A transplant was made October 23, and examined October 24, 26, and 29, 1915. No metachromatism was noticed. On October 29, I shook the tube vigorously to try to induce a heavy growth, which just began October 30. At examination at this time showed the large clubbed and branched forms and the immense bright red granules above referred to. When examined on an agar hanging block these forms showed very active growth and a fusion of the metachromatic granules which led Williams to interpret the process as a primitive sexual one.

FIG. 1

Strain 29 of my series also showed a remarkable series of pictures covering but two microscopic fields. The transplant was made to fresh potato, which developed a very granular organism from one that had the chromatin concentrated in the ends of the bacillus and which did not resemble the Klebs-Loeffler bacillus. It was stained in carbol-fuchsin and decolorized in the weak acid-alcohol above referred to and very lightly counter stained with weak Loeffler's. The very remarkable forms shown above were found at about one place in the cover-glass, due I believe to the fact that I barely touched the end of the wire on a very moist growth and drew it across the cover-glass but once. These forms are surely very suggestive of the karyokinetic figures so common in the higher forms.

MOTILITY

None of the cultures in my series showed motility and I have been able to find only one instance recorded in the literature.

[2] Schaudinn has shown a primitive conjugation in *B. Bütschlii*.

De Witt (1912) describes one such organism which she proved to be the cause of the pathologic condition from which it was isolated.

CAPSULES. SPORES

I have demonstrated capsules in my strain number I, isolated in coccus form from an animal. I have not been able to find any reference to such in the literature. There are many comments on the absence of capsules, spores and motility among the diphtheroids.

CULTURAL CHARACTERISTICS

Glucose-agar

The appearance of the colonies on this medium varies with the subgroup and very often with different strains of a subgroup. *B. xerosis* exhibits a dry, transparent film which gives the slant a ground glass appearance. This is not changed on prolonged incubation or by standing at room temperature. All observers agree on this point. *B. flavidus* also has a dry granular growth which may or may not be pigmented, but is usually much more luxuriant than *B. xerosis*. These are the only two subgroups of this species to give a dry growth.

Typically, diphtheroid colonies are very moist. Prochaska (1897) describes precise, whitish-grey colonies, which enlarge rapidly and form an elevated growth. They are dark in the center and clear on the border, which may be serrated. When pigment is present it varies from light or lemon-yellow, to orange or red. Some have a brown pigment. Hamilton (1904) has described a strain giving a purple color on agar. Prochaska (1897) calls attention to the fact that as the medium dries slightly the center of the colony may fade. My strains 4 and 5 faded out completely, even though the tubes were sealed. The growth became perfectly transparent. This is not a usual feature. Some strains refuse to grow on serum-free media when first isolated but can usually be trained to do so. The more saprophytic subgroups, *B. Hoagii* or *B. diphtheroides-liquefaciens*

give an abundant moist, coalescing growth which proceeds slowly at room temperature.

Blood agar

This medium is conducive to more luxuriant growth of all strains. The typical colony has a dark center and light border. Pigment is more likely to develop on blood-agar. Not infrequently the colonies are stippled. *B. flavidus* gives a yellowish, spreading, adherent growth which is wrinkled or corrugated radially. Some strains show concentric wrinkling. An occasional race is hemolytic. The bacillius of Preiz-Nocard and of Hall and Stone (1916) was markedly so. My strains 13 and 14 were slightly hemolytic. It is not uncommon for a blood-agar plate to become brown from the acid produced. Not infrequently the colony takes up the changed hemoglobin leaving the surrounding medium colorless.

Blood-agar plates offer a ready means of determining the purity of a culture on account of the various forms of colonies that develop. I have been able to separate different types of organisms by the type of colony produced. This is by no means an infallible criterion, since the variants of a pure strain may show different types of colony. It is often necessary to make several rapid transplants, when the colonies will then be alike in case the strain is pure.

Loeffler's blood serum

The various members of the group grow well on this medium, giving as a rule, moist, transparent or opaque colonies with the fastidious strains, and heavy, opaque, confluent growths with the saprophytic ones. There is little opportunity for colony differentiation with this medium. It is usually the best medium to indicate proteolytic change. De Witt (1912), Hamilton (1904), Wade and Harris (1915) and Graham-Smith (1908) have all described proteolytic strains of this group. One strain of my series (32) was a rapid liquefier of serum.

Gelatin stab

The majority of races will grow in gelatin, some of them quite luxuriantly, although it is the exception to have liquefaction occur. Most of the liquefiers are found in the subgroup *B. diphtheroides-liquefaciens*. Strains 4 and 5 of my series caused a slow liquefaction although they belonged to the *B. Hoagii* subgroup. Pigment formation is not uncommon in gelatin. Viability is well preserved. Strain 3 was alive after eighteen months on this medium. Ordinarily one sees a greyish-white growth which not uncommonly extends along the entire line of inoculation, inasmuch as this group readily adapts itself to anaerobic conditions. Strain 14 developed single, thin, spreading colonies along the upper third of the stab. They were dark in the center and had a lighter serrated border. Strain 21 developed a "nail-head" culture for a few days, then a funnel shaped liquefaction along the entire line of inoculation. Strains 31 and 42 grew mostly in the bottom of the tube showing their preference for anaerobic conditions.

Broth

Here again one finds great variation although there are two main types of growth; first, the formation of scanty, granular, flocculent or nebulous material that collects along the sides or bottom of the tube after twenty-four hours leaving a clear supernatant; second, the heavy, luxuriant, diffusely turbid growth, depositing a finely granular or mucoid sediment. Certain races develop a thin, friable pellicle which is deposited as a flocculent sediment on slight agitation of the tube. *B. xerosis* and *B. flavidus* more often give rise to a pellicle as they are more closely related to *B. diphtheriae* than the others. *B. enzymicus* grows very scantily in broth although it can be made to grow luxuriantly by the method described in Part I, Journal of Bacteriology, March, 1917. The broth has a foul or sour odor depending on whether acid or alkali is produced. De Witt (1912) alone has ascribed a peculiar odor to her cultures. She says that it resembles acetone, and was identical with that coming from the

lesions of the patient. I have observed a very unusual odor with strains 2, 11, 13, and 14. It is always noticed when a carbohydrate is fermented, and it resembles the odor of the esters of the higher alcohols. It resembles amyl alcohol very closely or the heavy sweet odor that comes from lilies. The fact that strains 13 and 14 were isolated from a case of gonorhoea suggests the idea that these organisms may be a factor in the repugnant odor of a case of this kind. It is known that the odor of the soil is often due to bacterial activity. It is interesting that all these strains belonged to the *B. enzymicus* subgroup.

Litmus milk

A majority of my strains caused but little change in this medium. The Ruediger bacillus (Ruediger, 1903) completely decolorizes litmus although no acid is produced. This feature constitutes the most noteworthy cultural characteristic of the organism. This is probably a reduction change. Several of Fox's (1915) gland diphtheroids reduce litmus. Strains 21 and 1 in my series exhibit this action. Graham-Smith mentions this change in his *B. diphtheroides-liquefaciens*. It most commonly occurs in saprophytic races producing alkali. Lactose is fermented as frequently as are some of the other sugars. This sugar is acted on pretty constantly by *B. diphtheroides-liquefaciens* and *B. enzymicus*. It is rarely acted on by the other sub-groups. When a coagulum is formed it is usually due to the production of acid, although the experiments of Fox (1915 b) and others indicate that certain strains have a coagulating enzyme. In many instances the amount of acid formed was not great enough to coagulate the milk unless it was boiled.

Alternation between red and blue was observed not infrequently in some of my strains which were feeble acid producers. This may perhaps be due to the possibility that there is a small percent of glucose in the milk which undergoes fermentation, the resulting acid entering into combination with some neutral or buffer substance in the milk, only to be thrown out again as the resultant alkaline fermentation displaced it. With two of my strains the litmus cleared and became of a bordeaux-red color.

Potato

The same variation which characterizes the appearance of the various races on other media holds true for this one. In general it may be said that the more luxuriant growers are adapted to this medium. Pigment is readily produced on potato. The growth is generally very moist or slimy and not infrequently is of a dirty grey color. The majority of Fox's (1915 b) strains show no growth on this medium and the same can be said of mine. However when used in the proportion in which it is found in Bordet's medium potato seems to exert a favorable influence on all forms. Particularly has this been true in the isolation of *B. Hodgkini*, but this form grows as well if not better on glucose-blood-agar.

MUTATION

Roux and Yersin and other of the earlier investigators claim to have been able to produce a diphtheroid from a Klebs-Loeffler bacillus by growing it at high temperature. Hewlett and Knight (Park and Williams 1910) Richmond and Salter (1898) claim to have transformed *B. Hoffmanii* into the Klebs-Loeffler bacillus; the former by culture and passage through guinea-pigs and the latter by passage through gold finches. Bergey (1904), Williams and others have not been able to obtain these results. Fox (1915 a) attempted mutation by culture on different lymph gland media but was unsuccessful.

THERMAL DEATH POINT DETERMINATIONS

Twenty-four hour cultures of salt suspensions of the bacilli were filtered through cotton-glass-wool in order to remove the clumps. The homogeneous suspension was then drawn into thin walled capillary tubes 2 mm. in diameter by 8 cm. long. These were placed in the water bath at varying temperatures for varying lengths of time and then cultured for one week in glucose-serum-broth. The results are indicated in tabular form.

TIME OF EXPOSURE	TEMPERATURE	STRAIN	CULTURAL RESULTS AT			
			55°C.	60°	65°	70°
minutes	°C.					
10	55°	T-337	+	−	−	−
5	60°	x-320	+	−	−	−
3	65°	x-323	+++	−	−	−
2	70°	1	+	−	−	−

It will be seen that 60°C. was fatal to various strains of this group under the conditions of the experiment. The antiformin-fast strain x-323 was not affected by ten minutes exposure at 55 while the growth of the others was markedly inhibited.

ANTIFORMIN TESTS

Salt suspensions of the various strains after being filtered through cotton-glass-wool were mixed with equal parts of antiformin and placed at 37.5°C. for two hours. They were then washed with NaCl twice, centrifuged in NaCl and the sediment stained by Gram and cultured on blood-agar. The results are shown in table 2. There seems to be the same variability regarding this test that is displayed by the group in other respects.

The *B. Hodgkini* strains seemed to yield more of the Gram-positive organisms in the sediment than did the other races which showed a tendency to be resistant to antiformin. None of them could be cultured following this treatment. It would not seem that antiformin resistant organisms are limited to any one subgroup of the diphtheroids, although this resistance becomes progressively less as we approach the saprophytic members. Hall and Stone (1916) report their *B. flavidus* as non-resistant to the reagent and Fox's (1915 b) observations tend to show that the reaction is non-specific.

OXYGEN

Most of the races are facultative anaerobes, as may be inferred from the cultural results on gelatin stabs. A few instances of

strict anaerobosis have been reported. Dick (1915) describes a Gram-negative and a Gram-positive strict anaerobe isolated from the urine of cases of chronic non-suppurative nephritis. Torrey (1916) has isolated an anaerobe from the lymph glands of Hodgkin's disease which he has called *B. lymphophilus*. Bloomfield tentatively makes a "partial pressure" group of diphtheroids. He says that they are slow growing and rather anaerobic. Rosenow has often noticed this quality in both diphtheroids and streptococci. Strain 2 of my series was isolated from the blood and urine as a strict anaerobe. Voigt has isolated strict anaerobes from the vagina, (personal communication).

SUMMARY OF MORPHOLOGY AND CULTURAL CHARACTERS

The diphtheroids readily adapt themselves to artificial media, this being more noteworthy in the subgroups *B. diphtheroides-liquefaciens*, *B. Hoagii*, and *B. Hoffmanii*. The more fastidious strains usually require the presence of serum in the medium. Viability is prolonged on all media, especially gelatin. The same strains may contain variants giving rise to different types of colonies which increases the difficulties of isolation. Blood-agar is a favorable medium for separating mixed cultures. In general, both cultural and morphological characteristics can be correlated with the sugar and complement-fixation tests.

FERMENTATION

Sugar fermentation has been regarded for a long time as one of the best criteria for the separation of bacteria into classes. On reviewing the literature on this subject I was astounded at the number of apparently contradictory results obtained. Further study regarding the large number of factors involved in this reaction, some of which are beyond control, together with the variety of methods used, caused me to accept most gratefully any uniformity that might be discovered.

Before citing the results of the various observers in this regard, a discussion of some of the difficulties regarding fermentative

reactions in this group might be in order. When it was first discovered that the Klebs-Loeffler bacillus produced acid in glucose broth and *B. Hoffmanii* did not, a distinction between the two organisms was based on this point. Gradually it was found that there existed other diphtheria-like organisms which fermented glucose but which were non-toxic. Accordingly, for a long time various observers described different strains of diphtheroids, making their observations only on glucose and attaching a sonorous name to the organism, which was based very often on some minor characteristics, most commonly, the morphological appearance. The small number of sugars used was the first difficulty in the way of a successful grouping of different strains.

When more sugars were employed, each observer had his own method of determining when fermentation had taken place. Even to the present time a variety of methods is used, many of them being very questionable for a procedure of this kind. Few observations have been quantitative. Litmus has been freely used as an indicator both in liquid media like Hiss serum water and in solid media as well. With scantily growing organisms like some of the diphtheroids it is very difficult to ascertain whether growth has actually taken place in the presence of litmus. It is also subject to reduction decolorization changes which are not infrequent in this group. But its greatest fault, particularly when used with the diphtheroids is its lack of delicacy. Generally speaking these organisms do not violently attack sugars, and many reactions which are positive to phenolphthalein are lost with litmus.

For example: Fox (1915 b) in studies as late as 1915 reports that "observations on carbohydrates were made on litmus-agar-sugar mediums of reaction neutral to phenolphthalein." In this case the organism would be compelled to produce at least 1.5 per cent acid before litmus would register it, a neutral phenolphthalein reaction being alkaline to litmus to the extent of 1.5 to 2.5 per cent. Since on many sugars, a variety of strains will not produce this amount of acid it is very easy to see how negative results might be obtained. Hiss serum-water with litmus as an

TABLE 3

Fermentation reactions

STRAIN NO.	SUCROSE	GLYCERIN	MANNITE	GLUCOSE	DEXTRIN	LACTOSE	MALTOSE	INULIN	SALICIN	RAFFINOSE	GAS	GROWTH
Sub-group B. enzymicus												
1	0.5	1.5	0.0	2.1	0.5	1.3	1.1	0.5	0.4	0.0	0	+++
2	0.5	3.4	2.7	2.8	0.5	0.4	0.7	0.5	0.0	0.4	0	+++
3	0.5	0.4	0.9	1.9	0.4	0.4	0.4	0.4	0.4	0.0	0	+++
9	0.9	0.3	0.4	0.7	0.3	0.4	0.3	1.2	0.0	0.0	0	++
11	0.6	0.3	0.45	0.9	0.4	0.4	0.5	0.8	0.5	0.6	0	++
13	0.0	0.65	0.0	1.5	0.4	0.45	2.3	0.3	0.3	0.3	0	+++
14	0.7	1.6	0.4	1.5	1.0	0.3	1.0	0.0	0.6	0.6	0	++
16	0.9	1.1	0.0	1.7	1.2	1.0	0.9	0.0	1.4	0.0	0	+++
33	0.4	0.0	0.6	0.8	0.7	0.4	0.4	0.3	0.0	0.0	0	++
x-323, 37	1.4	1.5	1.6	1.8	1.0	1.8	1.6	0.4	−0.4	0.9	0	+++
x-324, 38	1.2	1.2	1.4	1.5	0.9	1.2	1.2	−0.5	−0.4	0.0	0	+++
x-325, 39	1.6	2.5	2.2	2.8	1.5	1.4	1.1	0.0	0.0	0.0	0	+++
x-322, 40	2.6	2.8	2.5	2.8	1.8	2.6	2.1	1.1	1.1	1.0	0	+++
41	2.5	0.7	0.0	0.7	1.0	2.1	0.6	0.0	2.3	1.0	0	+++
42	1.7	0.8	0.0	2.0	0.5	2.7	1.2	0.0	0.0	2.0	0	++
x-319, 45	1.0	0.8	0.6	1.2	1.3	1.1	0.9	0.6	0.7	1.1	0	+++
Sub-group B. flavidus												
T-337, 35	0.0	0.6	−0.3	1.3	−0.4	−0.5	1.6	−0.6	0.0	−0.4	0	+++
Sub-group B. Hoagii												
4	0.5	0.0	0.0	0.5	0.0	0.0	0.0	0.9	0.0	0.0	0	++
5	0.4	0.0	0.0	0.3	0.0	0.0	0.0	0.4	0.0	0.0	0	++
6	0.8	−0.35	0.0	1.3	0.0	0.0	1.0	0.0	0.6	−0.3	0	+++
7	1.4	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0	+++
8	1.5	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.3	0.0	0	+++
19	2.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.3	0.0	0	+++
32	0.0	0.0	0.0	0.6	0.4	0.0	2.0	0.4	0.0	0.0	0	++
34	1.8	0.0	0.0	1.0	1.0	0.0	1.2	−0.5	1.0	0.0	0	+++
x-320, 43	0.0	−0.2	−0.3	0.7	0.0	−0.4	0.0	−0.4	0.0	0.0	0	+++
Sub-group B. liquefaciens												
21	−0.4	−0.3	−0.4	+1.1	−0.5	+0.4	0.0	−0.3	0.0	−0.3	0	+++
Sub-group B. Hoffmanii												
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
31	−0.5	−0.8	−0.7	−0.8	−0.4	−1.0	0.0	−0.6	0.0	−0.4	0	+++
x-321, 44	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
Sub-group B. Ruedigeri												
Sub-group B. zerosis												
12	0.0	0.0	0.6	0.7	0.4	0.0	0.0	0.0	0.0	0.0	0	++

indicator is often used but its disadvantages can be easily seen, and it should be displaced by more accurate methods. If one wishes to work with a medium of this sort, by far the most delicate and satisfactory is the one devised by Holman (1914) for streptococcic work. Andrade's reagent (basic fuchsin) which is the indicator used in this medium has the advantage of being very sensitive to the presence of acid. It is rather tedious to prepare however. Other observers have used neutral red in solid media which is also more delicate than litmus (Teoumin, 1913).

A third source of diversity is found in the length of time the cultures remain in the incubator. Variation in this regard has ranged from two or three days to an equal number of weeks. With a frankly alkaline or frankly acid producing organism this factor would not be of much moment, but with a group that at one time may produce acid and at another alkali in the self-same medium, it is obvious that it becomes an important factor for control if any uniformity is to be reached. Morse (1912 a) in her biometrical study of the diphtheroids has done more to correct this error than anyone else. She experiments with various strains on various sugars and finds that the sugars have a "time constant" for the production of maximum acidity which is different for every sugar. After this acid limit is reached, the organism attacks the protein of the medium and an alkaline reaction results. This has been shown by Kendall (1911) and Theobald Smith.

It should also be mentioned that adequate care must be taken to be sure that growth has occurred, as in its absence one would not expect fermentation. With doubtful strains this becomes a laborious matter in turbid or colored media. Chemically pure sugars should also be obtained in order to avoid another possible source of error. It is also important that sugars be fractionally sterilized as many of them are broken down by autoclaving.

It seems unnecessary to mention that the cultures tested should be pure. I have obtained a number of cultures from other sources which were free from organisms other than diph-

theroids but which contained more than one type of diphtheroid as was evidenced by the different sugar fermentations of the isolated strains.

Again it seems probable that certain races may at times lose their power of attacking certain sugars. I do not believe that this is a common occurrence especially outside of the body, but we have evidence that it does occur. J. H. Brown (personal communication) has definitely proved that such changes take place among the dysentery strains, and we have reasonable evidence that an organism may lose its power to ferment one sugar or acquire it for a sugar which it did not have, and still retain its other characteristics. *B. xerosis* is usually described as fermenting sucrose, yet I have a strain (number 12) typical in every other way and which on repeated tests did not ferment sucrose. Again Knapp (1904) finds a positive reaction to mannite while Zinsser is unable to verify the fermentation of that particular carbohydrate. Much more striking is the fact that *B. diphtheriae* is described as never fermenting sucrose, yet Graham-Smith (1908) has described a whole epidemic from which he obtained a sucrose fermenting true Klebs-Loeffler bacillus. The variability of the Klebs-Loeffler bacillus in this respect is well established.

Without doubt the best classification of the diphtheroids on the basis of sugar fermentations is the "Study of the diphtheria group of organisms by the biometrical method" by Morse (1912 a) As noted above she separated the diphtheroids into four main subgroups as follows:

(a) Group A is *B. Hoagii* which she names after Hoag who first described thoroughly a great many strains of this organism. She says that it ferments glucose and sucrose but not maltose. Regarding this last sugar she does not agree with Hoag since in his original article he states definitely that maltose is fermented and his tabular results bear out the statement. However this does not change the grouping. This organism was been described by more observers than any other of the diphtheroids except possibly *B. Hoffmanii.* About half of my *B. Hoagii* strains ferment maltose while the other half do not. On this basis it

will be very easy to place the so called *B. paralyticans* described by Ford-Robertson, (Morse, 1912 a). It ferments glucose, maltose and sucrose, and Morse is unable to place it on account of its maltose fermenting properties. It falls very nicely into one *B. Hoagii* subgroup. It can be very properly compared to the Klebs-Loeffler bacillus in its relation to lactose, and brings out a point which I wish to emphasize viz., that the fermentative history of this whole group shows that no hard and fast lines can be laid down, but that all the characteristics of an organism must condition its allocation.

(b) Group B is the second class of Morse' diphtheroids which she has named *B. flavidus*. Glucose is always fermented, maltose and glycerine usually but not sucrose.

(c) Group C or *B xerosis* always acidifies glucose and usually both maltose and sucrose.

(d) *B. Hoffmanii* is a nonfermenter.

Fox (1915 b) has studied a large number of strains from Hodgkin's glands as well as lymph glands from other conditions, particularly those draining enlarged joints. He tested the different strains on glucose, lactose, sucrose, maltose, mannite, glycerin, dextrin and galactose, but was unable to discover any striking regularities by this method. He did not work quantitatively and the other conditions which I have discussed on page 291 may contribute somewhat to the irregularity. However one seeming generality is noticed. The organisms isolated from the glands draining diseased joints have as a class a wider range of fermentation than the other strains. The possible significance of this fact I shall discuss later.

Hine (1913) attempts to classify the urethral diphtheroids by means of sugar reactions. He uses glucose, sucrose, maltose and lactose. Litmus is used as an indicator and seven days is the incubation period. The first class ferments no sugars (*B. Hoffmanii*); the second ferments glucose, sucrose and often maltose (*B. Hoagii*) and a third which he calls *B. coryza-segmentosus* ferments glucose, or glucose and sucrose. The urethral group proper ferments all sugars but lactose. These do not correspond to any of the other classes.

Torrey (1916) states that since no classification of the diphtheroids exists, he is not able to place accurately the various strains which he has studied, but forms three arbitrary groups for them based partly on sugar fermentation and partly on morphology. The vast majority of his strains fall in two common groups viz., *B. Hoffmanii* and *B. Hoagii*. From his brief description of some of his miscellaneous strains they resemble closely the *B. xerosis* or *B. flavidus* subgroup.

Teoumin (1913) studies the sugar reactions of 20 strains of pseudo-diphtheria. He has placed them in four classes yet they all have the same fermentative reactions, namely positive glucose and maltose. It is very evident that these groups correspond to Morse's *B. flavidus* and are identical with Hamilton's Group II.

DeWitt (1912) describes a pathogenic strain closely resembling *B. proteus* and producing both acid and gas in broth. Hamilton (1907) has also described several gas producing strains, and has studied 57 strains which fall into two groups. Her Group I had 40 representatives and fermented glucose and sucrose but was negative to maltose, lactose, and dextrin. This corresponds to Morse's *B. Hoagii*. Group II ferments glucose and maltose but never sucrose. Dextrin is 60 per cent positive and lactose 10 per cent. This corresponds to Morse's *B. flavidus*. Group III is the Ruediger bacillus which produces a soluble toxin but ferments none of the sugars.

It would seem that the following sugar fermentation method advocated by Theobald Smith is the best one which we have at present; and I have used it in my work. Broth rendered sugar free by previous treatment with *B. coli* is given a reaction of plus 0.8 to plus 1 to phenolphthalein. Each tube contains 9 cc. One cubic centimeter of a 10 per cent solution of the various sterile sugars is then added to each tube with a sterile pipette, and incubated for forty-eight hours to insure freedom from contamination. The sugars are titrated on the eighth day, as at this time, a maximum acidity is produced in maltose, mannite, dextrin, lactose, raffinose, salicin and inulin. If no acid is formed with glucose, sucrose or glycerin at this time, the

latter is again titrated at the sixteenth day and the former sugars at the twelfth or thirteenth day. This latter procedure was adopted following the suggestion of the Morse article. A control tube was always incubated when the reaction determined on the sugars was titrated, since it was found by actual experiment that a variation of between 0.3 and 0.4 per cent might be produced as the result of evaporation of the medium with concentration of the acid it contained.

Although this method is the most accurate and serviceable of any in use at the present time, it has some faults which it might be well to point out and which stand in a fair way to be corrected in the near future by the application of more accurate physicochemical methods. It has been known for some time that the titratable acidity of a medium is not its actual acidity but only forms a relative and often an inaccurate guide to an estimation of the actual concentration of H-ions contained therein. This idea has been worked out and elaborated very convincingly by Clark (1915). To begin with, he shows that the titrametric method of arriving at the reaction of a medium is inadequate as at present used. He points out that the only correct means of arriving at its true reaction is by an estimation of the H-ion concentration. All nutrient media contain in varying proportion the so called "buffer substances" of which peptone and phosphates are two familiar examples. These substances are able within limits to absorb acid as it is produced by an organism. The organism is thus protected from its action and is enabled to produce further acid. When titration is performed the free H-ions are neutralized and then dissociation of the buffer substance takes place yielding more H-ions. In this way the same organism might produce a much larger amount of titratable acid on one medium than on another. This is one way in which to account for the very large amount of acid produced by a number of Morse's strains.

Clark also criticizes severely the titration of media while hot. He claims that the enormous amount of dissociation resulting causes a variable reading.

W. T. Bovie (1913) has recently devised an ingenious method

for measuring the H-ion concentration. It will still be some time however before one is able to make such determinations with facility, but it indicates a great advance toward the solution of a problem which has given much trouble to all who have wished for a really accurate notion of the reaction of bacterial culture media.

I have used ten fermentable substances with the various strains in my series. The monosaccharide glucose; the disaccharides, sucrose, maltose and lactose; the higher saccharides, raffinose, dextrin, inulin and salicin; and the alcohols, glycerin and mannite. The results in the appended table 3 represent the percentage amounts of normal NaOH required to neutralize the acid developed by the organism. Phenolphthalein was used as an indicator. A minus sign preceding a number indicates alkali production. A special column for growth is shown which shows that any negative results were not due to the fact that insufficient growth was obtained. Plus three indicates a luxuriant growth plus two, moderate; and plus one a scant growth.

ANALYSIS

From a survey of the sugar reactions, it is seen at the outset that it will be neither possible nor necessary to exclude from a subgroup an organism which does not ferment precisely the same substances as the original strain after which the subgroup was christened. However desirable such a clean cut grouping might be, there is no criterion of classification among these organisms which will be rigidly constant. I have already indicated a great many reasons why this must be so, and have substantiated my contention with evidence collected from the literature.

None of my strains were aerogenic. Some strains produced no acid on any of the sugars, while others had a wide fermentative range. Between these extremes one finds all gradations. Quantitatively the diphtheroid group does not produce a high percentage of acid, although some of Morse's strains compare favorably

with the amount produced by the streptococci. Torrey (1916) also describes an anaerobe producing a very high amount of acid.

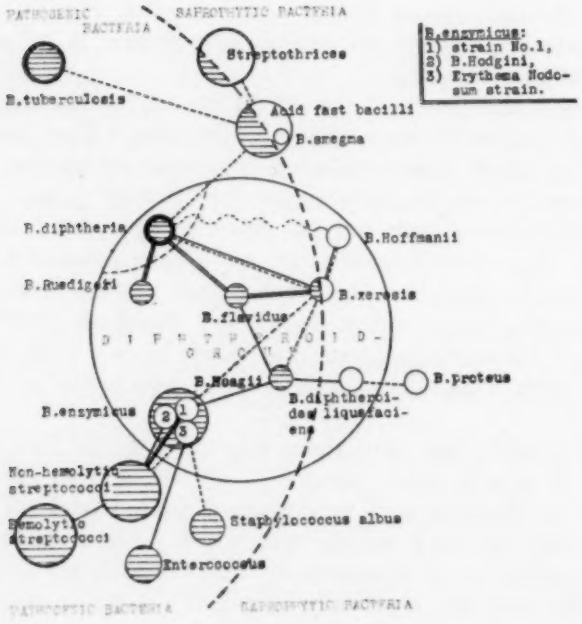
Six of the strains were *B. Hoffmanii*, 10, 24, 25, 31, 43, and 44, producing no acid from any sugar, but often yielding an alkaline reaction. Strains 4, 5, 7, 8, 19, 34, acidified glucose and sucrose constantly and in some cases salicin or inulin. Strain 34 also fermented maltose. These strains correspond to *B. Hoagii* of Morse. One strain, 12, fermented glucose and dextrin and was obtained from Dr. Wolbach's laboratory as *B. xerosis*. This organism usually ferments sucrose also but did not do so in this case. It was typical in all other respects. Strain 32 might be considered a variant of *B. Hoagii*. Strain 35 fermenting maltose and glucose was typical in all respects of Morse's *B. flavidus*.

Strains 1, 2, 3, 9, 11, 13, 14, 16, 33, 37, 38, 39, 41, 42, and 45, although isolated from a variety of sources, I have placed in one group which I have called *B. enzymicus* on account of the wide range of sugar fermentation which they possess. It is certain that these organisms fall into a group which is different in many ways from the other groups. They ferment from six to ten sugars which is a phenomenon that has been commented on only once before to my knowledge. Rosenow (1915) mentions that his erythema nodosum strains have a wide range of fermentation although he does not say how many sugars are fermented.

It is possible that this group may have to be further subdivided at a later time. Strains 37, 38, 39, and 40 were given me by Dr. Bunting and were isolated from Hodgkin's glands and in addition were strains with which he had produced results in animals. It is noteworthy that qualitatively and quantitatively they are practically identical. Strain 3 is a strain of my own isolated from Hodgkin's glands which has nearly the same reactions as Bunting's strains. The remaining characteristics of this subgroup have been discussed in Part I, Journal of Bacteriology, March, 1917. Strain 45 (one of Bunting's strains from pseudoleukemia) ferments all ten sugars and is thus fairly closely related to the Hodkgin's strains in that it

is a powerful fermenter. However the Hodgkin's strains do not ferment inulin and salicin although strain 37 attacks raffinose slightly. The chronic leukemia strain number 44 and the Banti strain number 43 ferment none of the sugars, and on this basis must fall into the *B. Hoffmanii* class or the *B. Ruedigeri* class. The latter however has a soluble toxin. These strains are both from Dr. Bunting.

CHART NUMBER 1.



In Chart I, I have indicated diagrammatically the interrelationships of the various subgroups and their connection with the Klebs-Loeffler bacillus, as well as the relation of the entire diphtheria group to other micro-organisms. The broken curved line bounds a segment of a circle which represents the

domain of the pathogenic forms. Since there can be no hard and fast line separating the pathogenic and saprophytic fields, the division is pictured by a broken line. The larger area surrounding the arc of this circle represents the domain of the saprophytic organisms. The entire diphtheria group is shown by the medium sized circle occupying the border line position, part lying in the saprophytic field and part in the pathogenic.

The individual subgroups of diphtheroids are shown by small circles, and are shaded or not according as they fall within or without the pathogenic field. A double solid line connecting the subgroups indicates a close relationship; a single solid line indicates a moderate linking and a dotted line a distant relationship. A curved line indicates a mutation.

Beginning at the small area indicating the *B. diphtheriae* there is shown a close direct relationship between it and *B. flavidus*. If there is any organism which could properly be called an avirulent diphtheria bacillus this is certainly the one. I believe that this designation should be applied to an organism similar in most respects to the Klebs-Loeffler bacillus but lacking the specific toxin formation of the latter. In the dry growth on blood and serum media, the firmness of the colonies and their adherence to the media in old cultures, *B. flavidus* is much more like the diphtheria bacillus than are most of the diphtheroids. It also forms a very distinct, friable pellicle on glucose-broth which falls to the bottom on slight agitation of the tube. Involution forms appear in the older cultures. It ferments glucose, maltose, and glycerin, but not sucrose. It is pathogenic for guinea-pigs but does not produce the lesions of the diphtheria bacillus. Most noteworthy was the evidence of complement fixation tests, (see Part III). The fact that this strain was practically alone in its separation from the other strains was noticeable. It is quite closely related to *B. xerosis* (strain 12), a fact that Morse's complement fixation work has also brought out. Hamilton (1907 b) also believes this glucose-maltose fermenter to be closely related to the true diphtheria bacillus. Four of the seven strains which she studied killed guinea-pigs. She describes it as Group II of her classification of the diphther-

oids, but refers to it as *B. xerosis*. Morse has identified Hamilton's Group II as *B. flavidus* of her own classification. The strain which I have studied is one that Teacher (1915) has shown to be the causative organism in a epizoötic of infective abortion in guinea-pigs. To reiterate briefly: One finds the cultural, morphological, immunological and sugar reactions of *B. flavidus* to be strikingly similar to those of the true diphtheria bacillus, but inasmuch as it produces no specific toxin it must be classed among the diphtheroids.

Closely related to the diphtheria bacillus as well as to *B. flavidus*, is *B. xerosis*. It also has a scant, dry, adherent growth and a diphtheria-like morphology. It is described as characteristically a sucrose fermenter, although the strain which I have studied has no action on this sugar. Its complement-fixation and agglutinin reactions show that it is closely related to *B. flavidus*. It has only been described by one observer as having been pathogenic for guinea-pigs (Eyre, 1896), producing only local edema, decided loss of appetite and weight. Escherich and C. Frankel (1896) and F. Schanz (1896) have all noted the strong resemblance between the avirulent Klebs-Loeffler bacillus and *B. xerosis*. The only reference to its pathogenicity in man is the report of Eyre (1896) who isolated it from 15 cases of chalzion, and reproduced the condition in the eyes of animals. Accordingly I have given it a very close relationship to both the Klebs-Loeffler bacillus and *B. flavidus*.

B. Hoagii is an entirely different type of organism from the previous two described. It is a luxuriant grower on almost any kind of media, under almost any conditions, and has the widest distribution of any of the subgroups. Hoag describes it as fermenting glucose, sucrose, and maltose. He has isolated 150 strains of it. The so called *B. paralyticans*, supposed by Ford-Robertson and others to have an etiological relationship to paresis is merely the saprophytic *B. Hoagii*. It has the same sugar reactions. Hamilton (1907) has also studied 40 strains of this organism wrongly calling it the psuedo-diphtheria bacillus. She also finds that it ferments only glucose and sucrose.

My strains 4, 5, 19, and 34 were representatives of this sub-

group. Strains 4 and 5 fermented glucose, sucrose and inulin, while 19 fermented salicin in place of inulin; strain 34 in addition to glucose, sucrose and maltose fermented salicin and dextrin. A study of the characteristics of these organisms shows that they all belong to the same group, despite the fact that the fermentation varies somewhat. They have in common the fermentation of glucose and sucrose. In this connection, I wish to mention strain 32 which has the same fermentative powers as strain 34 except for a doubtful reaction with sucrose. Even glucose is fermented only to 0.6 per cent. In addition it liquefies serum agar (Loeffler's). In spite of these differences I consider it to be merely a variant of *B. Hoagii* and not deserving of some special name. Recourse to the immunological reactions of the two strains seems to bear out the contention. *B. Hoagii* is also related to strains 1, 2, 3, and x-323 which subgroup is located at the other extreme. For these reasons I regard this subgroup as occupying an intermediate position between *B. flavidus* on one hand and *B. enzymicus* on the other. When pathogenic, it is usually found in connection with suppurations, usually not initiating a pathologic process, but sustaining it when once started by a more virulent organism, Hamilton (1907), Bergey (1904). Experimentally I have produced local subcutaneous abscesses in guinea-pigs with *B. Hoagii* after the animal had previously received an intraperitoneal injection of the same organism.

I have next to consider an organism which I believe merits a distinctive position. Ruediger (1903) and later Hamilton (1904) have described a very important bacillus, which morphologically resembles the diphtheria bacillus, but culturally and fermentatively seems more closely allied to *B. Hoffmanii*. It grows heavily on agar, and gives diffuse cloudiness in broth. Its most noteworthy cultural characteristic is its decolorization of litmus. This is probably due to its reducing action which is possessed to a greater or less degree by most members of the group, although complete decolorization of litmus is not usual especially in the absence of acid formation. This form ferments no sugars but produces a soluble toxin which is very pathogenic

for guinea-pigs. The toxin is not neutralized by anti-diphtheritic serum. I was not able to obtain a culture of this organism but Hamilton's immunological work indicates that it is distinct from *B. Hoagii* and *B. flavidus*. I have given it a separate place closely related to the diphtheria bacillus, and more distantly related to *B. Hoffmanii* and have named the subgroup *B. Ruedigeri* after the author of the original description of this organism.

B. Hoffmanii, I have considered as a mutant in accordance with the opinion expressed by Flexner and others. Its total fixing value is only eight, showing that the cross-reactions with the other sub-groups are not great. It has slight cross-fixation with *B. enzymicus*. Kolmer (1916) has shown that there is a definite cross reaction between this organism and *B. diphtheriae*. Strains 10, 24, 25, 31, 43, and 44 of this series belong in this group.

A very interesting bacillus was described by Graham-Smith (1904) in 1904 which he has called the *B. diphtheroides-liquefaciens*. He first isolated it from the mouth of a patient supposed to be suffering from diphtheria. It is characterized by the following features. The organisms are very long and curved and may be motile. There is practically no segmentation or involution produced; slow liquefaction of serum and gelatin; slow but abundant growth on potato. Glucose, or glucose and lactose are fermented and milk usually coagulated. Litmus is decolorized. Nitrates are reduced and much indol is formed. No gas is produced.

Hamilton (1904) has described an organism which can be placed in this subgroup but which produces gas in glucose broth. Klein (1903) has described a *B. diphtheroides* which also liquefies both serum and gelatin, produces hepatization in rat's lungs and is pathogenic for guinea pigs. De Witt's (1912) proteus-like organism may also be included in this group. Strain 21 of my series must also be placed here. It liquefies gelatin but not serum, ferments glucose and lactose and coagulates milk with decolorization of the litmus. This assemblage of organisms is very closely related to *B. Hoagii* (strain 34) but particularly to strain 32 which is a variant of strain 34. These sub-

groups have a distinct relationship to the saprophyte *B. proteus* a fact which I have shown in the diagram.

I now come to a subgroup which I have designated *B. enzymicus* on account of the range of its sugar fermentation. Its distinctive characteristics can be learned in detail by a study of strain 1 which I take to be a representative member of the subgroup.

It has three main characters not common to the other subgroups: first, a remarkable pleomorphism; second, a wide range of sugar fermentation; and third, a distinct biological relation to the streptococci.

On account of these qualifications I have represented this subgroup as being more intimately connected with the non-hemolytic streptococci than with the other members of the diphtheroid group. This particular strain produces in addition a poisonous substance in the broth filtrate which resembles in many ways a true toxin. Strain 1 from a purely morphological standpoint can be called either a diphtheroid bacillus or a non-hemolytic streptococcus. It can be made to grow in long chains of diplo-streptococci. Biologically it has the features of a streptococcus of a moderate grade of virulence in that it constantly produces suppurative arthritis, cholescystitis, and myositis in animals. Reference to the immunological reactions shows how definite is its relation to the non-hemolytic members of this group. The viability of this strain is much more prolonged than that of the streptococcus and its virulence is also lost much sooner on artificial media. It then occupies an intermediate position between the remainder of the diphtheroid group and the streptococci.

Rosenow's erythema nodosum strain appears to show almost identical cultural and morphological features, as well as pathological, although he has not attempted to demonstrate its relation to the streptococci. However he has suggested it. It can be placed with certainty in the subgroup *B. enzymicus*. Likewise, I believe that the so called *B. Hodgkini* can be placed in this subgroup, although if it is demonstrated to have the etiologic relation to Hodgkin's disease that is now claimed, it will no

doubt merit a more specific ranking. The strains in this series which I have studied and placed in this group are strains 1, 2, 3, 9, 11, 13, 14, 16, 33, 37 (x-323), 38, 39, 41, 42. Without exception the strains in the bacillary form were very scant growers, even on blood or serum media. I have been able permanently to alter the morphology of most of them, and in every case to develop quickly a very luxuriantly growing strain from one cultivated with difficulty.

I shall later describe the relation of this group to the enterococcus of Thiercelin and the complement-fixation tests show some cross-reaction with the staphylococcus. This work in connection with that of Frankel and Much who interpret *B. Hodgkini* as a special form of the tubercle bacillus, is evidence that the group touches on the more definitely acid-fast organisms such as the *B. tuberculosis*. Gordon (Graham-Smith 1908) has shown the relation between certain streptothrices and the diphtheria group. As a result of this evidence, I have placed as contiguous, the *B. tuberculosis* and the streptothrices, as well as the enterococcus, the streptococcus, the staphylococcus and *B. proteus*.

Any of the diphtheroid subgroups under favorable conditions may become pathogenic, although this proclivity is much more marked in some than in others. *B. enzymicus* containing as it does examples of the erythema nodosum, streptococcoid, and *B. Hodgkini* strains would seem to represent an adaptable assemblage which can easily become pathogenic. Likewise we have many examples of the pathogenicity of *B. flavidus* and *B. Ruedigeri*. On the other hand it is very rare to find *B. Hoffmanii* pathogenic, although Fox (1915a) and others have reported it as the probable cause of certain benign unilateral types of pharyngitis.

I shall now consider the various diphtheroids which have been reported from time to time under various names and relate them to this classification so far as possible. Cautley (1894) described an organism which he calls *B. coryza-segmentosus* which he believed to be the cause of some of the common colds. Gordon (1901), Benham (1906) and Graham-Smith (1908) have

isolated various strains of it. It ferments glucose, galactose, lactose, levulose, sucrose and maltose. Benham has renamed it *B. septus*, on account of its morphology at the particular time when he happened to observe it. It corresponds well to strain 16 of my series isolated from the nasal discharge of a fresh cold, and strain 11 isolated from a normal throat. It falls under *B. enzymicus* or the group of active fermenters. We know that it is not related to coryza except as a secondary invader. Neumann (1902) from a study of 206 cases of nasal colds, believes that diphtheroids have no etiological relation to the condition. He found them in 98 per cent of diseased noses, and 100 per cent of normal noses.

Graham-Smith (1908) has isolated a diphtheroid which he calls *B. maculatus*. Its poor growth on most media is its most noteworthy characteristic. It ferments glucose. From this general description it probably falls into the *B. xerosis* or *B. flavidus* subgroup. He says that it resembles closely a non-virulent diphtheria bacillus, in which case it would naturally fall in the latter group. I see no reason for giving it a separate name. *B. muris* described by Klein (1903) is an interesting organism. It was isolated from the hepatized lung of a white rat, and he was able to reproduce this lesion in other rats. Nothing but a large local abscess was produced in guinea-pigs. Culturally and morphologically this form has a strong resemblance to the true diphtheria bacillus. In the absence of a more complete description it could be placed in the *B. flavidus* subgroup. Bergey (1904) and Dean (Graham-Smith 1908) also isolated organisms from abscesses in a leprosy-like disease of rats which have the same characters as Klein's *B. muris*. MacFadyean and Hewlett (Graham-Smith 1908) have isolated the same bacillus from the mouth of healthy and diseased pigeons.

Nakanishi (1900) and Brown (Graham-Smith 1908) isolated an organism from cases of vaccinia and variola which they called *B. lymphae-variabilis*. It is easily identified as belonging to the subgroup *B. Hoffmanii*. Galli-Valerio (Graham-Smith 1908) has called his organism recovered from vaccine lymph, *Corynebacterium vaccinae*. This also is *B. Hoffmanii*. Klein

describes the *B. xerosis variolae*, and *B. albus variolae* from glycerin emulsions of small-pox crusts which are in all probability the ordinary *B. xerosis*. The descriptions are inadequate. De Simoni (Graham-Smith 1909) isolated an organism from the same sources which corresponds to *B. Hoagii*. Levy and Fickler (1900) isolated a *Corynebacterium lymphae-vaccinalis* which is very probably *B. flavidus*.

Graham-Smith's *B. xerosis canis* is merely the ordinary *B. xerosis*. His *B. diphtheriodes gallinarum* is very probably *B. flavidus* and his *B. ceruminous* is the common *B. Hoagii*. His *B. diphtheroides-brevis* and *citreus* also have nothing distinctive and can be grouped under *B. Hoagii*. Graham-Smith has also isolated a *B. auris* from the ear. De Simoni (Graham-Smith 1908) has found the same organism there, while Griffith has isolated it from the normal eye. Bergey has isolated an identical organism from the vagina which was pathogenic for guinea-pigs in large doses. All these organisms can be grouped under *B. Hoagii* although the sugar reactions are only given in part. Certainly there is nothing distinctive about any of them.

Bloomfield (1915) divides the diphtheroids of lymphatic glands into three arbitrary groups, although there is really no biological classification made. Group I he correlated with surface saprophytes, probably meaning *B. Hoagii*, *B. Hoffmanii* and *B. xerosis*. In Group II he describes a short pleomorphic bacillus mildly anaerobic, and another type resembling a coccus with the same characters as the short pleomorphic bacillus. They are probably identical, one being the coccus form of the other. It probably would be placed with *B. enzymicus*. Group III is heterogenous.

Hine (1913) has classified urethral organisms most of which he has identified with *B. coryza-segmentosus*. As I have already suggested, all these organisms can be grouped under *B. enzymicus*. His Group I is *B. Hoffmanii* and his Group II is *B. Hoagii*.

Torrey studied the lymph gland diphtheroids and placed them in three arbitrary groups. The majority of the strains which he places in Groups I and II can be identified with *B. Hoagii* and *B. Hoffmanii* from their action on sugars. Some

strains in class I belong to *B. enzymicus*. In class III *B. xerosis* or *flavidus* predominates. He describes a *B. lymphophilis* which I have not had opportunity to study. It is an anaerobe obtained mostly from Hodgkin's glands and ferments glucose, glycerin and sucrose. He believes it to be a distinct species.

The classification of Teoumin (1913) based on sugar reactions and agglutination tests I have already referred to on page 294. These organisms fermenting maltose, levulose and glucose plainly belong to the *B. flavidus* subgroup. Wolbach and Honeij (1914) describe a diphtheroid bacillus which they believe to be identical with the majority of those described from leprosy. It is a very typical example of *B. flavidus* in its morphological and cultural characteristics and in its sugar reactions.

De Witt (1912) has described a very unusual pathogenic diphtheroid organism which she thinks is closely related to *B. proteus*. It liquefies both serum and gelatin, is actively motile, produces gas in glucose broth, coagulates milk and develops indol. This form is closely related to *B. diphtheroides liquefaciens* of Graham-Smith and undoubtedly will fall in this subgroup.

(To be continued)

THE FUCHSIN-ALDEHYDE REACTION ON THE ENDO MEDIUM

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Endo (1904) suggested the use of a medium of the following composition for the differentiation of *B. typhosus* from *B. coli*-like organisms:

Neutralized nutrient agar (3 per cent agar)	1000 cc.
Chemically pure lactose	10 grams
Alcoholic solution of fuchsin	5 cc.
Ten per cent sodium sulfite solution	25 cc.
Ten per cent sodium carbonate solution	10 cc.

The medium had a light pink color when hot and was practically colorless when poured into Petri dishes and allowed to harden. On this medium Endo observed that *B. coli* and its close allies gave a distinct red coloration while *B. typhosus* was practically colorless. The red color of the *B. coli* colonies, according to this author, was due to the formation of acid but he did not give any experimental evidence to substantiate this belief.

Harding and Ostenberg (1912) have shown that the reaction is due to aldehyde formation. These authors suggest, also, that by the substitution of other carbohydrates in place of lactose in the Endo medium this reaction might be used as a means of differentiation of the colon-typhoid-dysentery group in place of acid production.

Robinson and Rettger (1916) have stated more recently that the red coloration of colon colonies on Endo medium is the result of acid production and not of aldehyde formation. These

[1] The author desires to acknowledge the valuable assistance of Dr. R. E. Buchanan and Prof. Max Levine in the preparation of this paper.

authors quote Grey as having found an aldehyde (acetaldehyde) produced in his fermentations only under anaërobic conditions.

It is the purpose of this report to explain, if possible, the discordant results by a comparative study of some of the reactions with acid and basic fuchsin which have been decolorized with sodium sulfite and bisulfite and to compare these reactions with those obtained when the true Schiff's fuchsin-aldehyde reagent[2] is employed.

REACTIONS WITH ACID AND BASIC FUCHSIN DECOLORIZED WITH SODIUM SULFITE

Saturated aqueous solutions of acid and basic fuchsin were decolorized with sodium sulfite. With these solutions qualitative tests with concentrated acids and 40 per cent formaldehyde were carried out. Four or five drops of the test substances were added to five to seven cubic centimeters of the reduced fuchsin solution. A pink color was taken as positive. The results are shown in table 1.

TABLE 1

Reactions of acids and formaldehyde with acid and basic fuchsin decolorized with sodium sulfite

	HCl	H_2SO_4	CH_3COOH	$CH_3CHOHCOOH$	HCHO	HCl AND HCHO
Acid fuchsin.....	++	++	+++	+++	+++	+++
Basic fuchsin.....	−	−	++	+	−	+++

From table 1 it is to be seen that the acid fuchsin showed no marked differentiation between aldehyde and acid although the mineral acids did not show quite so strong a red color as was produced by the organic acids and formaldehyde. With the basic fuchsin the mineral acids produced a straw colored solution. The organic acids showed a slight reaction which might be produced by traces of formaldehyde as an impurity and, perhaps also, by the formation of a strong base and weak

[2] According to Mulliken Schiff's fuchsin-aldehyde reagent is prepared by dissolving 0.2 gram basic fuchsin in 10 cc. freshly saturated solution of sulfur dioxid. After standing for some hours the solution should be a straw color. This is then made up to 200 cc. with distilled water and kept in a dark place.

acid combination, e.g., sodium lactate, which will give a reaction with the reduced fuchsin. The reaction with formaldehyde was negative. With an acid also however, a very strong reaction was obtained. This showed that the coloration with formaldehyde is dependent upon an acid medium.

Endo plates were poured using basic fuchsin decolorized with sodium sulfite. A loop of different dilutions of hydrochloric, sulfuric, acetic and lactic acids was placed upon the medium and allowed to incubate at 37°C. for twenty-four hours. The twenty-fifth, fiftieth and one hundredth normal acids were used and in no case was a pink or red color produced. If, however, a loop of formaldehyde and a loop of fiftieth normal acid (any one of the four acids used), were added a very strong reaction was given. A loop of 40 per cent formaldehyde alone did not produce a red coloration upon the plate.

From the experiments outlined above, it is to be seen that acid fuchsin decolorized with sodium sulfite does not differentiate sharply between aldehyde and acids. The basic fuchsin shows a coloration with concentrated organic acids, but none with concentrated mineral acids. Formaldehyde alone does not give a reaction but when an acid is present a pronounced red color is produced.

REACTIONS WITH ACID AND BASIC FUCHSIN DECOLORIZED WITH SODIUM BISULFITE

Acid and basic fuchsin were decolorized with sodium bisulfite and qualitative tests with concentrated acids and 40 per cent formaldehyde were carried out as above. The results are shown in table 2.

TABLE 2

Reactions of acids and formaldehyde with acid and basic fuchsin decolorized with sodium bisulfite

	HCl	H_2SO_4	CH_3COOH	$CH_2CHOHCOOH$	HCHO	HCl AND HCHO
Acid fuchsin.....	++	++	+	+	+++	+++
Basic fuchsin....	−	−	+ (later decolorized)	+ (later decolorized)	+++	+++

The acid fuchsin as before did not differentiate sharply between acid and aldehyde. The organic acids did not produce quite so strong a color as the mineral acids. The basic fuchsin did not give a permanent red color with either mineral or organic acids. Formaldehyde gave a very strong coloration without the addition of acid because the bisulfite contains an acid radical which will serve to carry out the reaction. The results upon the Endo plates were the same as with the fuchsin reduced with sulfite solution. This is explained by the neutralization of the acid by the addition of the sodium carbonate solution in the Endo medium.

The most important point which the experiments with the sulfite and bisulfite show is that an acid radical is necessary for a strong red coloration when an aldehyde acts upon the reduced basic fuchsin.

REACTIONS WITH BASIC FUCHSIN DECOLORIZED WITH SULFUR DIOXID (SCHIFF'S FUCHSIN ALDEHYDE REAGENT)

With Schiff's reagent acids did not give any coloration, although acetic acid gave a slight pink color possibly due to traces of aldehyde. Strong caustics, potassium and sodium hydroxide gave a slight pink, due, as the chemists have shown, to the formation of a strong base and weak acid combination. Strong ammonium hydroxide did not give a pink color. Lactic acid neutralized with sodium carbonate gave a light pink.

Robinson and Rettger reported that concentrated lactic acid as well as dilute mineral acids gave a red color upon an Endo plate. It had been observed in this laboratory that the concentrated acid gave a red color with the formation of gas from the excess sulfite and carbonate, but no color was produced by any acid which approximated the concentration of the acid produced by *B. coli*. These statements applied only to decolorized basic fuchsin, however, as a strong color may be obtained with acid fuchsin when tested with either concentrated or dilute acids. It is possible, therefore, that these authors used acid fuchsin in their Endo medium; at least their results could be easily explained upon this hypothesis.

DO BACTERIA PRODUCE ALDEHYDE?

A number of organisms were grown in a medium of 0.5 per cent carbohydrate, 0.5 per cent peptone and 0.5 per cent dipotassium phosphate and were tested for aldehyde formation with Schiff's fuchsin aldehyde reagent. All cultures were incubated at 37°C. and tested in twenty-four hours and seventy-two hours. The results are given in table 3.

TABLE 3

Production of aldehyde in a medium containing 0.5 per cent each of dipotassium phosphate, peptone, and carbohydrate

	GLUCOSE		LACTOSE*	
	24 hours	72 hours	24 hours	72 hours
B. acidi-lactici 131	+	+	+	+
B. acidi-lactici 135	+	+	+	+
B. communior A. 123	+	+	−	+
B. communior A. 19	+	+	+	+
B. communior B. 144	+	+	+	+
B. communis B. 125	+	+	+	+
B. aerogenes	+	+	+	+
B. cloacae	+	+	+	+
B. pyocyaneus	−	−	−	−
B. pneumoneae	+	+	+	+
M. roseus	−	−	±	+
B. subtilis	+	+	−	−
B. prodigiosus	+	+	−	−
B. phosphorescens	−	−	−	−
B. typhosus (1)		+		−
B. typhosus (2)		+		−

* The lactose medium did not give a test for monosaccharides after sterilization.

It is evident from the above table that the colon-aerogenes group will produce aldehyde when grown in a sugar medium. Since the concentration of acid, produced by *B. coli*, upon the Endo plate is in itself not sufficient to produce a pink or red color the typical *B. coli* reaction must of necessity be due to the formation of both acid and aldehyde.

SUMMARY

Aldehyde formation by many bacteria may be demonstrated by Schiff's fuchsin-aldehyde reagent in glucose and lactose media.

Decolorized basic fuchsin reacts with aldehydes only in the presence of an acid to give the red color of the fuchsin-aldehyde test.

The red color of the *B. coli* group upon the Endo plate is due to the production of aldehyde together with acid.

Acid fuchsin which has been decolorized with sodium sulfite or bisulfite will give a red coloration with either acid or aldehyde. The color may be discharged by the addition of alkali.

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